BLOOD COAGULATION

HUMAN BLOOD COAGULATION

AND ITS DISORDERS

by

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(a)



(b)



(c)

PLATE 1

Electron microscope photographs of fibrinogen and fibrin

(a) Electron micrograph of the margin of a dried droplet of fibrinogen solution to show the particulate non fibrillar character of the dried material
(b) Electron micrograph of a mixture of thrombin and fibrinogen showing linear arrangement of particles (protofibrils) and the development of unit fibres apparently from the lateral union of several protofibrils
(c) Micrograph of fibrin with the formation of compound fibres *Fine fibres lie in parallel with coinciding densities giving a striated appearance to large fibres*

These figures were shadowed with gold In unshadowed preparations the cross striation of compound fibrin fibres is a striking feature (from van Zandt Hawn and Porter 1947 and Porter and van Zandt Hawn 1949)

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He thought he saw an Argument
That proved he was the Pope
He looked again and found it was
A Bar of Mottled Soap
A fact so dread he faintly said
'Extinguishes all hope!'

LEWIS CARROLL

PREFACE

In producing a book on blood coagulation there is a risk of falling between two stools On the one hand it is difficult to make a book sufficiently advanced in time and thought to interest the established workers in the subject, while on the other it is likely to be too voluminous and erudite for the clinician pathologist or physiologist who though not primarily interested in coagulation would like to know what developments are taking place

Being aware of these difficulties we have tried to avoid them with what degree of success remains to be seen The book presents for instance a number of hitherto unpublished experiments which particularly those relating to the new concept of plasma thromboplastin may interest other workers There are descriptions of the technical methods that must be used in the investigation of clotting defects and attempts to explain their rational basis (or the lack of it) which may be useful to pathologists Nearly half the book is given up to descriptions of the clinical states which arise from or produce defective clotting and to the problems of their diagnosis and treatment. Lastly any general survey of research on coagulation provides for the student of the scientific method magnificent examples of the dangers of incomplete experiments and unjustified conclusions The fact that we ourselves have probably fallen into such pitfalls as often and as heavily as anyone else does not detract in the least from the value of the lessons that can be learnt from such a survey

Much of the cost of the experimental work described has been met from grants from the Medical Research Council and from the Nuffield Foundation Haematological Research Fund We wish to thank Dr E. Bidwell and Dr A S Douglas for their great help in reading both the manuscript and the proofs and also Miss Evelyn Beer for the line drawings We are grateful to Professor L J Witts

PREFACE TO THE SECOND EDITION

The progress in research on blood coagulation made during the past four years has involved extensive rewriting of the original text of this book in order to make the second edition an up-to-date account of the subject. The first edition appeared just as the importance of the thromboplastin generating system of the blood was beginning to be recognized since its publication advance in knowledge resulting from this recognition has begun to gain impetus The separation of Christmas disease from haemophilia and the use of the thromboplastin generation test in the diagnosis and prognosis of a number of clotting defects and in the assay of individual clotting factors are all recent advances which we hope are covered by the revision and expansion of the corresponding sections of this second edition

But inevitably the speed of research outstrips the efforts of authors printers and publishers A significant growth of knowledge in blood coagulation seems now to be taking place along biochemical lines Just as the study of the thrombin-fibrinogen reaction has revealed a biochemical basis for the action of thrombin so a study of thromboplastin generation is beginning to provide a biochemical basis for part of the early stages of coagulation It is possible that the chemical identification of the lipoid coagulation factors may soon be achieved If the nature of these factors and of any reactions which they may undergo can be established this will mark the beginning of a new era in research on normal coagulation abnormal bleeding and the problem of thrombosis

We wish to express our gratitude to Dr Ethel Bidwell for her advice in preparing the manuscript and for her help in reading the proofs We thank Professor P A Owren for reading the manuscript of Chapter XVIII and making valuable suggestions We are grateful to the publishers of the *Journal of Physiology* and to Drs W R. Merz and M Wiener for permission to publish Tables 10 and 40

for permission to describe two cases The following authors editors and publishers have kindly allowed reproduction of illustrations Drs van Zandt Hawn and Porter, and the editors of the *Journal of Experimental Medicine* (Plate 1) Dr Bessis and the editors of *Blood* (Plate 2a) Drs Braunsteiner Febvre and Klein and Messrs Grune and Stratton (Plate 2b) Drs Grégoire and Florkin and the editors of *Physiologia Comparata et Oecologia* the editor of the *Journal of Clinical Pathology* (Figs 10 47 and 48) Dr Owren and Messrs Grune and Stratton (material in Table 12) Messrs Grune and Stratton Heinemann J B Lippincott and Charles C Thomas the editors of the *Journal of Biological Chemistry* the *Archives internationales de physiologie Archives internationales de pharmacodynamie (et de thérapie)* the *Journal of the Missouri State Medical Association* the *Acta medica Scandinavica* and to Drs P H Owren A J Quick P Nolf W H Seegers A G Ware J H Olwin and J L Fahey for permission to quote passages of published works

PART ONE

CHAPTER I

THE GROWTH OF KNOWLEDGE OF BLOOD COAGULATION

INTRODUCTION

Until about fifteen years ago interest in blood coagulation was mainly restricted to the relatively few people who were actually engaged on its investigation This does not mean that the volume of work on this subject was small it was enormous Probably no other biological process of similar extent has been so continuously and intensively investigated Throughout the world there have grown up schools of workers for whom the study of blood coagulation has been their life s work. Even in 1863 Lister in his classical paper which demolished the current ammonia theory of clotting wrote that coagulation has engaged the best energies of many very able men so that it might well seem presumptuous in me to hope to communicate anything new regarding it Since that time new contributions have appeared with an ever increasing velocity and in the year 1953/54 over 600 papers on blood coagulation were published

To the uninitiated, the fascination that this work has for its devotees must seem difficult to understand. But a number of explicable factors operate to develop this attraction so that once involved in it the new recruit is almost powerless to draw back To begin with the spontaneous transformation of fluid blood to solid clot has a stimulating effect on the curiosity like that produced by a well-executed conjuring trick. One feels that so simple and striking a phenomenon must have a simple and striking explanation, and the urge to discover it is immediate Next, there is the deceptive ease with which the work can be started All that is needed is a few glass tubes and a watch a supply of blood and ingenuity Later the more mechanically minded investigator will discover that almost equally good results can be obtained by apparatus of limitless complexity for coagulation lends itself to being recorded by viscometric, photometric nephelometric and potentiometric methods complete of course with the latest mechanical and electronic developments Thereafter experiments are easy to plan

concerned with many subsidiary developments such as synthetic resins and water repellent surfaces

The high flights of academic research have now come down to earth so to speak in every hospital laboratory almost in every doctor's consulting room The pathologist whether he likes it or not may be expected to carry out and presumably to understand, investigations in blood coagulation that would have been considered erudite by the most advanced of the academic workers a few years ago Prothrombin determinations by one or two stage methods and prothrombin consumption tests are routine procedures and the heparin resistance test, tests for anticoagulants and the assay of antihaemophilic globulin may on occasion legitimately be required Unless they are experts in this field the clinician and the pathologist are liable to become bewildered when they seek the theoretical foundations on which these tests are based and from which their results can be judged The existing literature is not very conducive to clarity of thought on the part of the new reader in fact some of its ingredients would be more digestible by the habitual consumer of crime fiction The number of aliases false clues conflicting statements and fallacious deductions to be found there would arouse the envy of the most prolific writer of detective novels

It is the modest purpose of this book to attempt to reduce this profusion to the smallest possible residue of useful and sober facts to sort out the different names which really belong to the same thing the different things which have had the same name the things which probably do exist from the things which probably do not and to present what is known about the probable ways in which the things which remain react together to produce a clot At each stage some account will be given of the interpretation placed on their results by individual workers and an attempt will be made to emphasize those interpretations which account for the observations of different groups of workers In this way theory can be simplified because although fifty or more substances have been proposed by various authors there is general agreement about relatively few In a work of this sort much selection and omission is inevitable and many admirable contributions may seem to be ignored This cannot be avoided if blood coagulation research is to be presented as a coherent sequence of progress in knowledge

From this restricted and we hope simplified basis the significance

and to carry out and provide unlimited opportunities for being most satisfyingly clever Endless experimental combinations and permutations can be worked through with an excitement that would be familiar to the player of roulette or the follower of football pools Always it seems the next will be the winning combination revealing the one clue that has been missed by everyone else the flash of illumination that will show the long sought simple explanation of blood coagulation Undaunted by the perpetual postponement of this revelation the investigator enters the last stage of his evolution as a coagulation worker and becomes a maker of theories He may become so enamoured of these children of his imagination that he loses sight of the facts on which their existence was founded, and fails to recognize new facts which might indicate their abandonment He argues he pours out a flood of literature he defends his theories against all comers He founds another school of blood coagulation research

There are of course more serious reasons for embarking on this work Blood coagulation has an obvious if at present unspecified importance in the haemostatic mechanism It is a common observation that people with defective blood clotting may die of haemorrhage or lead lives of almost perpetual disability A proper understanding of coagulation and the faults to which it is liable is of real importance and the search for the cause and cure of haemophilia actuated much of the work done during the past century It is a measure of the complexity of the problem that these limited and practical objectives have not been reached For many years the results of coagulation research could only be described as literary and philosophical and its practical contributions to medical science were limited to the introduction of citrates and oxalates as useful anticoagulants and the diagnostic recognition of the long clotting time in haemophilia The ordinary doctor therefore was able to regard with tolerant detachment and the examination candidate with only temporary dismay the almost ludicrous growth of confusion in this subject

During the last few years this position has completely changed Blood coagulation research has led to the discovery of a new vitamin the recognition and treatment of a whole range of newly defined haemorrhagic diatheses it has raised practical problems in genetics and eugenics introduced with all their attendant problems anticoagulant drugs for the treatment of thrombosis and has been

of the other components The value of an hypothesis was defined by Newton in 1672 when he wrote For the best and safest method of philosophizing seems to be first to inquire diligently into the properties of things and of establishing those properties by experiments and then to proceed more slowly to hypotheses for the explanation of them For hypotheses should be subservient only in explaining the properties of things but not assumed in determining them unless so far as they may furnish experiments The mere contemplation of phenomena without ideas is not scientific investigation But if facts without ideas are blind ideas without facts are sterile In blood coagulation the ideas have tended to get out of hand their main purpose the stimulation of experiment being often forgotten In consequence the literature is full of sterile hypotheses which because they cannot be proved or disproved lead to no further experiment and worse still full of hypotheses which masquerade as facts and are assumed by the uncritical reader to represent real knowledge There has been a tendency to postulate the existence of a new factor whenever a phenomenon is observed which cannot be readily explained and later it is often difficult to distinguish between what is established and what is postulated

The value of hypotheses can best be illustrated by a line of research which had results of great practical importance the discovery of prothrombin In 1845 Buchanan observed that when the fibrin from clotted blood was washed a coagulant substance was present in the liquid which would clot hydrocele fluids Schmidt (1861 1862 1892) confirmed these findings and observed that an active coagulant, fibrin ferment or thrombin could be extracted from serum by alcohol precipitation On the other hand if fresh unclotted blood was run into alcohol no similar substance could be isolated These observations led to the suggestion that an active coagulant appeared in the blood during clotting This was a most useful hypothesis it explained the observed facts and immediately led to further experiments It was found for example that well washed extracts of tissues which promoted the clotting of cooled blood would not clot the fibrinogen made from plasma by the salt precipitation method of Hammarsten (1877 1899) whereas the fibrinogen was rapidly clotted by Schmidt s fibrin ferment from serum It therefore seemed unlikely that the fibrin ferment came from the tissues and it was postulated that an inactive precursor of the fibrin ferment was present in the plasma an idea giving rise to the first version of the

of the more useful tests can be better appreciated, and some further understanding of the pathological states due to defective coagulation may be gained It will be complained no doubt that even this restricted presentation is depressingly complicated Some will say as they always have said that these factors co-factors activators and inhibitors are probably artefacts or mere figments of the imagination But each of the clotting factors which are presented as entities in this book has, by its simple absence from the blood produced a specific haemorrhagic diathesis in man Even in these days of multitudinous deficiencies we have yet to see so objective a manifestation as haemorrhage produced by the simple deficiency of a figment

THE DIFFICULTIES OF RESEARCH IN BLOOD COAGULATION

The present confusion is partly due to a failure on the part of some workers to realize that the investigation of blood coagulation is not easy as it deceptively appears at first sight but extremely *difficult* *Long processes of evolution lie behind the clotting* mechanism and its superficial simplicity is akin to the simplicity of genius The blood contains within itself not only the clotting factors that will initiate accelerate and limit coagulation as required but also a series of safety devices to prevent coagulation occurring within the vessels and for dissolving fibrin which is no longer useful

The main difficulty in the investigation of this system is the extraordinary restriction of the available approach Fibrin formation is the only phenomenon that can be used as an indicator by the experimenter With the possible exception of platelet agglutination no other observable change physical or chemical takes place during coagulation In consequence everything that is learnt about coagulation has to be inferred from the occurrence of fibrin formation It is now recognized that this process is the end result of a long chain of reactions and each link in this chain must be studied by its effect on the final stage The further removed the observation is from this stage the more indirect must be the information

THE PROBLEM OF THE HYPOTHESIS

In any scientific investigation the sequence of observation hypothesis experiment and inference form the structure by which new knowledge is gained In blood coagulation the peculiar nature of the problem has favoured the growth of the hypothesis at the expense

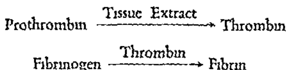
same and if these different names do refer to one substance which interpretation of their activity is the more probable Initially this confusion is unavoidable but the progress towards simplification in terminology would be facilitated if all scientists could appreciate the inherent uncertainty of their own conclusions and the probable value of the experiments of other workers

A different terminological confusion has arisen from the unavoidable limitation of knowledge existing at a particular time Mellanby (1909 and 1930) isolated a mixture of proteins from plasma by dilution and acidification to pH 5 3 Mellanby called this fraction fibrinogen though it is now clear that it contains at least three other factors of importance prothrombin Factor V and antihaemophilic globulin Mellanby thought that fibrinogen and prothrombin were closely associated factors and he could not be expected to recognize the presence of factors which had not been discovered. Other workers who held the view that the clotting of fibrinogen was independent of factors other than thrombin used such complicated substrates as oxalated plasma or plasma treated with $Ca_3(PO_4)_2$ and called them fibrinogen solutions though fibrinogen was actually only one of several active components liable to complicate their experiments Other workers came to the conclusion that the active principle of brain extracts was cephalin and having accepted this hypothesis felt justified in referring to crude tissue extracts as cephalin' in their published works

Supposedly Pure Factors

The isolation of fractions from plasma has led to many interesting and important experiments Using isolated prothrombin it was possible to show that prothrombin was converted quantitatively to thrombin Similarly the role of accelerators of blood clotting could not have been studied without some attempt to isolate the accelerating substances But two difficulties arise from the use of these isolated substances The substance isolated from plasma may bear little resemblance to its prototype in plasma The process of isolation may split off some essential part of the molecule This difficulty can never be avoided entirely Then the substance isolated from plasma is pure in a very limited sense A factor may be freed from constituents recognized by a particular worker but it cannot be known to be free from unrecognized substances Thus prothrombin prepared by modern methods is free from antithrombin the

so-called classical theory of blood coagulation (Schmidt 1892 Morawitz 1905) This may be written



This theory involved five coagulation factors and of these thrombin fibrinogen and fibrin had already been isolated from plasma or clotted blood and were generally accepted as real entities Prothrombin on the other hand, was a hypothetical substance which was not and could not be generally accepted at the time that it was proposed The hypothesis of the existence of prothrombin was fruitful because it led to very many subsequent experiments In 1909 Mellanby isolated a substance from plasma which though not coagulant itself could be converted into a coagulant Eagle (1935a) and later in 1947 Owren demonstrated that the amount of thrombin formed was directly proportional to the amount of this precursor present It is because the observations of very many workers can be explained by the existence of prothrombin that it can now be accepted as a real entity in blood coagulation though the properties attributed to it have varied according to the view of blood coagulation currently held

THE SOURCES OF CONFUSION

Multiple Terminology

Terminology may be a very important source of confusion in blood coagulation work Coagulation factors when they are first proposed have a tenuous claim to reality and nothing but the accumulation of confirmatory evidence over many years can improve their position A factor in transition towards general recognition may cause chaotic confusion The conception of the factor may vary with the work of individual experimenters and the same factor may have misleadingly varied names For example serum has been said to contain serum prothrombin *conversion accelerator* Factor VII convertin serum accelerator globulin the Christmas factor plasma thromboplastin antecedent and co-thromboplastin (Chapter V) These terms are based on the interpretation of experimental results and it is far from simple to know whether all of these factors are distinct entities or if they are the

of the plasma contained two clotting factors only prothrombin and fibrinogen In 1911, Addis made a series of observations on the clotting defect in haemophilia which are as brilliant today as they were when first published. He found that the clotting of haemophilic blood was accelerated by the addition of Mellanby s fraction from normal plasma, even after the fibrinogen had been removed from it Addis accepting as a fact Mellanby s inference about the composition of this fraction himself inferred that it was the normal prothrombin which corrected the clotting defect in haemophilia He then made the hypothesis that the clotting defect in haemophilia was due to an inactive state of its prothrombin The hypothesis which is put forward as a conclusion of his paper was accepted as a fact by subsequent workers and resulted in considerable confusion When it was shown that the prothrombin in haemophilia is normal Addis work was forgotten. Not for many years was it known that the normal globulin fraction contains besides prothrombin and fibrinogen other clotting factors and in particular the antihaemophilic factor If Addis had stated his observations rather than his hypothesis in his conclusions work on haemophilia would probably have been advanced by twenty-five years

Hypotheses are frequently worded in such a way that they appear to be statements of fact and when made by respected authorities are often accepted as such It has been said that platelets do not contain thromboplastin but supply the activator which converts inactive plasma thromboplastin (thromboplastinogen) to an active state (Quick 1950a) This statement refers to a hypothesis and not to a fact Or *They* (Ware and Seegers 1948a and b) *have found that the accelerator is present in both plasma and serum but it has different characteristics in the two media* the plasma type being the inactive or precursor form and the serum type the active form of the factor that is the real accelerator of prothrombin conversion (Olwin and Fahey 1950) The part of this statement in italics refers to observation and the latter part to hypothesis In the original work Ware and Seegers were careful to separate observation and hypothesis but in reporting by their associates the distinction is less obvious Or It was also demonstrated then that factor V is an active substance a precursor or proenzyme which is activated during the clotting process (Owren 1950a) This statement also is hypothetical and not a statement of observation, despite the misleading use of the word demonstrated Thus in the work of the three greatest modern schools of

antihaemophilic globulin Factor V and fibrinogen, but this does not imply the isolation of a functionally pure substance

The Measurement of Coagulation Factors

A substance cannot be studied scientifically until it can be measured but how can it be possible to measure a substance whose very existence is in doubt? Moreover a method for the measurement of a substance may be both reasonable and useful in one era of blood coagulation research but the interpretation of the results of the test may be revolutionized by new experiments Thus in 1935 Quick's one-stage test was both a great advance in coagulation technique and also thought to be a reliable method for measuring prothrombin Today Quick's test is still a valuable tool though it is to be doubted if it often gives a measure of prothrombin The value of conclusions from a test must depend on existing knowledge and as this advances previous conclusions must be reviewed

Unnecessary confusion may also arise from sheer carelessness Thus there are two common methods of expressing the results of the one-stage prothrombin test in which the final figure is expressed as a percentage of normal These two methods bear no numerical relation to each other and yet in written communications the method selected is often not specified

Confusion of Fact with Inference and Hypothesis

In blood coagulation research the processes of observation inference and making hypotheses are so closely inter-related that it is sometimes difficult to separate them Yet it is essential that the distinction should be made If an experiment is carefully carried out the reagents clearly specified and the results lucidly described the author has contributed a statement of *fact* which is of lasting importance The logical *inferences* drawn from such observations may appear to be valid at the time of the experiment but may be shown to be fallacious at any later time by an extension of knowledge Making a *hypothesis* is not a logical process it is an effort of the imagination going beyond facts and inference into supposition In itself a hypothesis can provide no evidence or even a basis for argument but only a plan for future action

The way in which these things can be mistaken for each other is best illustrated by a number of examples In 1909 Mellanby carried out experiments from which it was inferred that a globulin fraction

classical theory have dealt with a limited aspect of the subject they will be considered under separate headings

THE THROMBIN-FIBRINOGEN REACTION

The thrombin-fibrinogen reaction has been studied by many workers partly at least because it is the simplest of all coagulation reactions The properties of thrombin and fibrinogen have been studied and the reaction between the two has been observed with the electron microscope It has been shown by many workers that when thrombin is added to fibrinogen the clotting time of the fibrinogen is inversely proportional to concentration of thrombin. Methods for the measurement of thrombin and fibrinogen have been devised Using a method for the measurement of thrombin it has been possible to study thrombin formation in plasma and in mixtures of factors isolated from plasma

THE FORMATION OF THROMBIN

Prothrombin

As already stated prothrombin was a hypothetical substance when first proposed. Its establishment as a real coagulation factor depended on two lines of approach Firstly if prothrombin is removed from plasma then no clotting occurs even if all other factors are present in optimum proportions Thus Bordet and Delange (1914) showed that prothrombin could be removed from plasma by adsorption with $Ca_3(PO_4)_2$ and the supernatant plasma from this adsorption often called adsorbed plasma would not clot on the addition of tissue factor and calcium If some of the substance removed from the $Ca_3(PO_4)_2$ by elution was added to the adsorbed plasma clotting occurred rapidly These invaluable experiments which have been confirmed on innumerable occasions demonstrated that clotting can be prevented by the removal of a substance and initiated by its restoration. This work laid the foundation for the second line of approach to the problem the isolation of prothrombin from plasma This isolation following the method of Bordet and Delange has been effected by Nolf (1928 1945) Owren (1947) Fantl and Nance (1948a) Ware and Seegers (1948a) etc A natural sequel to the isolation of prothrombin was the demonstration that the amount of thrombin formed is directly proportional to the amount of prothrombin present (Eagle 1935 Herbert 1940 Owren 1947 etc.) Recently Seegers McClaughry and Fahey (1950) have shown that

blood coagulation research it is not always easy to make the fundamental distinction between what is actually found and what is suggested which in itself shows how difficult it may be to make the distinction In spite of this difficulty it is essential that an attempt should be made to distinguish observation from deduction and hypothesis

A hypothesis based on previous research is the clue which stimulates experimental work A good hypothesis is therefore one which is unambiguously worded, which is in keeping with all previous facts and which arouses sufficient interest to stimulate further experiments to discover more facts According to this view the hypothesis is the beginning rather than the end of an experiment and the hypothesis should be stated at the beginning rather than at the end of a scientific contribution If this were done the confusion might be less and other workers might be able to discover the observed facts from published communications with less difficulty

It may be relevant to finish this philosophical argument with a quotation from an author whose reports are seldom concluded with hypotheses

'The pool of blood coagulation is well stocked Anybody can pull up what looks like a fish But if 'the fish is later examined dispassionately it often reveals itself to be a frog indeed sometimes a winkle We once caught a fish that proved to be sphagnum It is our practice to attempt to keep the journal volume down by first ascertaining if the fish' caught six or twelve months ago – can be caught again – and to find out what happens on curing Even with this practice we find it difficult to discard all the frogs winkle and sphagnum (Link 1944)

THE CLASSICAL THEORY OF BLOOD COAGULATION

The outline of the work leading to the discovery of prothrombin which forms the basis of the classical theory of blood coagulation has already been summarized It is not proposed to deal in detail with the earlier controversies on blood clotting but to give some account of the great mass of work which confirmed the classical theory and then to indicate very briefly some of the dissident theories with which this subject has been beset from its earliest stages Since the majority of the studies which have confirmed the

view is supported by Eagle and Harris (1937) Tagnon et al (194-), Tagnon (194-) and Ferguson (1949) who showed that proteolytic enzymes could form thrombin from prothrombin and by Macfarlane and Pilling (1946a) and Macfarlane (1947) who showed that soya-bean trypsin inhibitor which is anti-proteolytic inhibits the action of tissue extracts On the other hand Mertz Seegers and Smith (1939) held that the tissue factor is required quantitatively for thrombin formation This problem will be discussed in Chapter IV

Platelets

Bordet and Delange (191-) recognized that platelets have thromboplastic activity and these authors often used platelet extracts (cytozyme) as a source of thromboplastin More recent study has suggested that while platelets contribute an important thromboplastin component their activity is different from that of brain extracts They require to react with plasma components which are not required by brain extracts This problem will be considered in greater detail in Chapter VI

Calcium

Since Arthus and Pagès (1890) observed that calcium precipitants inhibited coagulation an effect that was reversed when calcium was added few have doubted that calcium is required for coagulation Schmidt thought that calcium was required for the reaction between fibrinogen and thrombin but Hammarsten (1899) showed that this was not so Morawitz (1905) therefore recognized that calcium must be involved in the initial phases of blood clotting

Vines (1921) and Quick (1940) found that greater amounts of oxalate were required to prevent coagulation than should be necessary to neutralize all the ionized calcium present. This fact has suggested that the calcium taking part in blood coagulation is present in a combined form Ferguson (1937) suggested that a calcium cephalin compound was formed Quick (1940) believed that calcium was incorporated in the prothrombin molecule Wohlisch and Paschkis (1924) have shown that if coagulation is prevented by $MgSO_4$ and the ionized calcium removed by dialysis no clotting will occur unless calcium is added Sabbatini (1900) demonstrated that relatively small amounts of calcium are required for clotting Wohlisch (1929) suggested that the gross excess of oxalate may be necessary to depress ionization to this level

thrombin can be formed from prothrombin even if significant amounts of all other recognized coagulation factors are absent Prothrombin therefore appears to be the one essential precursor of thrombin existing in the plasma and this under optimum conditions is converted to thrombin quantitatively The practical importance of this line of research was increased by observations which lead to the belief that several haemorrhagic states were caused by prothrombin deficiency

The Tissue Factor

The role of tissue extracts in blood coagulation was recognized many years ago when it was found that exudates rich in cells coagulate more readily than those with few cells Bird plasma collected with special precautions and freed from cells remains fluid indefinitely but coagulates rapidly on the addition of a trace of tissue juice It has now been shown that the coagulation of mammalian blood can be delayed very considerably by the removal of cells and products of cell breakdown (Cramer and Pringle 1913 Brinkhous 1947 Quick 1947a etc) The tissue factor differs from thrombin in that it resists boiling by which thrombin is destroyed and although tissue extracts accelerate the clotting of plasma freed from tissue products they do not contain thrombin because they will not clot fibrinogen prepared free from prothrombin

The chemical nature of the tissue factor has been investigated Bordet and Delange (1913) thought that thrombokinase (or cytozyme) was a lipoid of the lecithin group Gratia and Levene (1922), Wadsworth Maltaner and Maltaner (1927, 1929 and 1931), Tocantins (1944a and b etc) and many other workers considered that the lipoid was cephalin However Fischer and Hecht (1934) showed that increasing purification of cephalin progressively decreased its thromboplastic activity Howell (1912) emphasized that while alcoholic extracts of tissues resist boiling aqueous extracts which are powerfully thromboplastic lose their activity on heating He suggested that in the tissues the lipoid component is associated with a protein and the isolation of a purified lipo-protein thromboplastin by Chargaff et al (1944) supports this view

The mode of action of the tissue factor has also been studied Many workers have held that it is an enzyme which accelerates thrombin formation but which does not affect the amount of thrombin formed and is not consumed during the reaction This

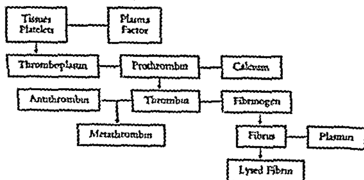
some part in maintaining normal fluidity of intravascular blood Heparin is not present in measurable amounts in normal blood but appears in large amounts in anaphylactic shock following peptone injections after exposure to ionizing irradiation and occasionally determines a haemorrhagic diathesis (Waters Markowitz and Jaques 1938 Jaques and Waters 1941 Crisalli and Cotlessa 1950)

FIBRINOLYSIS

Following surgical operations haemorrhage adrenalin injections and the secretion of adrenalin as a result of fear blood collected clots in the normal way but if the clots are allowed to stand at 37 C the fibrin is dissolved. Nolf (1908 1928 etc.) believed that the agent responsible for lysis an enzyme of plasma origin later called fibrinolysin or plasmin played some part in normal coagulation Lenggenhager (1946) also holds this view for he claims that a plasma fibrinolytic enzyme (thrombocatalysin) liberates thrombokinase (thrombokinin) from prothrombokinase (prothrombokinin)

Whether or not this enzyme really plays any part in coagulation is undecided but it is reasonable to suppose that a fibrinolytic enzyme may be important in dissolving intravascular clots

All the evidence outlined above supports and elaborates the classical theory of blood coagulation According to this theory prothrombin is a single substance which is quantitatively converted to thrombin under the influence of thromboplastin and calcium Fibrinogen is converted to fibrin by thrombin and the speed of the reaction is controlled by the amount of thrombin present From all of this work a coherent view of coagulation emerged which explained the great majority of observations



INHIBITORY SUBSTANCES

Schmidt (1892) recognized that thrombin disappeared rapidly from serum after coagulation being converted to 'metathrombin' and claimed that thrombin could be restored by the action of acids and alkalies Morawitz (1905) and later Mellanby (1909) showed that weak solutions of thrombin clotted fibrinogen more readily than oxalated plasma Quick (1938) showed that this antithrombic activity was associated with the albumin fraction of plasma Astrup and Darling (1942) studied the mechanism of inactivation of thrombin by antithrombin and showed that this was a slow progressive process Klein and Seegers (1950) have shown that the antithrombin is capable of neutralizing enormous amounts of thrombin Lyttleton (1950) has shown that the inactivation process is complex and cannot be explained in terms of simple first or second order interactions

The discovery of heparin by McLean (1916) was followed by an increasing interest in anticoagulant substances Howell and Holt (1918) found that the action of heparin was dependent on a fraction of plasma proteins precipitated between ⅓ and ½ saturation with ammonium sulphate Howell considered that heparin inhibited the formation of thrombin and based his theory of blood coagulation on this belief Mellanby (1935a) and Quick (1938) showed that heparin was mainly an inhibitor of the thrombin-fibrinogen reaction in plasma It has little effect on the thrombin-fibrinogen reaction taking place between pure solutions of thrombin and fibrinogen its activity depending on association with a protein in the albumin fraction of plasma The chemical composition of heparin was clarified by Jorpes and Bergstrom (1937) The isolation of heparin in a relatively pure form led to its extensive use in the treatment of thrombosis

Collingwood and MacMahon (1912) first postulated the existence of an antithromboplastin in plasma and Tocantins (1943a and b 1944a and b 1945) and Tocantins and Carrol (1949a) who have given evidence in support of its existence hold that it is present in excess in haemophilia Tocantins and Carrol (1949a) have isolated an inhibitory substance from plasma which they consider to be an antithromboplastin The evidence for the existence of this substance is still more or less indirect and until further information is available it must be considered to be a hypothetical substance

The role of these inhibitory substances in normal coagulation is still in doubt, but it is reasonable to suppose that they may play

THE TISSUE FACTOR ACTS AS A COAGULANT OF FIBRINOGEN

The early recognition of the importance of tissue extracts has naturally led to various interpretations of their role in blood coagulation Wooldridge (1883 1889) and later Mills and Guest (1921) have held that the tissue factor acts as a direct coagulant of fibrinogen Wooldridge postulated two types of fibrinogen fibrinogen A in the tissues and fibrinogen B in the blood The direct combination of these two led to clotting Fibrinogen A occurred in the plasma as well as in the tissues and was a part of plasma which separated out on standing in the cold This fraction may correspond to the plasma thromboplastin of other workers Wooldridge was aware that tissue extracts would not clot fibrinogen prepared from plasma by salt precipitation but he held that this isolated fibrinogen was an artefact which bore little functional relationship to his plasma fibrinogen B Wooldridge s theory had the disadvantage that it was not susceptible to experimental proof

PROTHROMBIN IS DERIVED FROM THE TISSUES

Nolf (1908) and Fischer (1934) both thought that prothrombin was derived from the tissues Nolf suggested that it arose from leucocytes and endothelial cells and Fischer that it was derived from muscle These theories are contradicted by the isolation of prothrombin from cell-free plasma and the demonstration that the tissue factor is not consumed during clotting This view was probably encouraged by the difficulty in preparing tissue extracts free from plasma

THE THEORY OF STUBER AND LANG

Stuber and Lang (1930) observed that glycolysis appeared to run parallel to blood clotting and that substances which increased or decreased glycolysis shortened or lengthened the coagulation time and concluded that glycolysis promoted coagulation This theory rested on the barest analogy and the assertions on which it is based have been denied by Hartmann and Kuhnau (1930) who showed that plasma freed from sugar clotted with $CaCl_2$ and that in haemophilia glycolysis proceeded at a normal rate though coagulation was delayed This hypothesis served little purpose since it failed to explain observed facts and led to no useful experiments

THE THEORY OF HOWELL

Howell (1935) who investigated the inhibitors of blood coagulation in detail held the view that prothrombin was converted to

This theory made possible quantitative methods for the measurement of prothrombin The development of these methods was encouraged by the clinical importance of prothrombin deficiency in jaundice and newborn infants and the use of dicoumarin to produce hypoprothrombinaemia for the treatment of thrombosis

The techniques in use are all based on the classical theory and they may be said to be the culminating point in its usefulness The methods have proved invaluable clinically but have introduced difficulties which the classical theory cannot explain In the one-stage method of Quick (1935a) it is assumed that the *speed* of thrombin formation in the presence of optimum proportions of thromboplastin and calcium is proportional only to the amount of prothrombin present As will be seen later this assumption is not true in plasma the speed of thrombin formation is affected by factors other than prothrombin In the two-stage technique proposed by Warner Brinkhous and Smith (1936) it is assumed that the *amount* of thrombin formed is controlled entirely by the amount of prothrombin present This assumption is also invalid The two techniques often give conflicting results because each records a different aspect of the coagulation process and neither measures prothrombin specifically The voluminous controversies about these methods have arisen from the fact that the theory on which they are based does not account for all the phenomena of blood coagulation

DEVIATIONS FROM THE CLASSICAL THEORY OF BLOOD COAGULATION

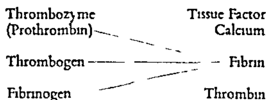
The outline of experimental work which has supported the classical theory of blood coagulation gives an impression of steady progress along a single line of argument In fact throughout the history of this work there have been many dissident views Some of these diversions have been useful in that they were based on valuable experiments and stimulated others Some of the observations could not be explained in terms of the classical theory and foreshadowed the modern chaos which has resulted from attempts to modify the classical theory Many of these theories will be outlined because no picture of blood coagulation would be complete without some appreciation of the theoretical difficulties which beset the worker in this field.

bogen is necessary This is present in excess in plasma but has not been isolated The formation of fibrin is due to the union into a single complex of thrombozyme thrombogen and fibrinogen When mammalian plasma is treated with a gelatinous precipitate of $Ca_3(PO_4)_2$ or $Al(OH)_3$ the main part of the thrombozyme is removed leaving behind the fibrinogen and proteins of hepatic origin

In 1945 Nolf s views are expressed in more modern terms

'The classical theory according to which thrombin is formed from a single plasma substance prothrombin is contradicted by a study of plasma adsorbed with $Ca_3(PO_4)_2$ and the proteins which adhere to the precipitate From this study it is clear that the prothrombin of the plasma is not a single substance but a mixture of two substances which can be separated one from the other by $Ca_3(PO_4)_2$ Both are proteins one is absorbed by $Ca_3(PO_4)_2$ (thrombozyme) and the other remains dissolved in the supernatant adsorbed plasma (thrombogen)

From these extracts Nolf s view of blood coagulation may be expressed as follows



The essential feature of this theory is the belief that a factor in addition to the classical prothrombin is necessary for the clotting of fibrinogen

THE THEORY OF BORDET

Bordet s (1920) theory like that of Nolf was put forward to explain a number of experimental findings which could not be accounted for by the classical theory of blood coagulation It was observed that the blood of birds collected into paraffin lined containers clotted more slowly than that collected into glass tubes This fact suggested that some substance present in the circulating blood was altered by contact with glass Bird and rabbit plasma freed from

thrombin by calcium alone and that in the circulating blood this reaction was prevented by a combination of heparin and prothrombin In the presence of thromboplastin the heparin was inactivated and the prothrombin was freed to react with calcium This theory immediately suggested further experiments Insignificantly small amounts of heparin can be found in normal blood (Monkhouse, Stewart and Jaques 1949) Mellanby (1935a) and Quick (1938) have shown that heparin in association with the albumin fraction of plasma inhibits the thrombin-fibrinogen reaction Mellanby (1935a) showed that thrombin was formed in the presence of heparin

THE THEORY OF NOLF

The theory of Nolf is in rather a special category Nolf s views on blood coagulation anticipated the findings of later authors and some of his experiments are clear and simple and have been confirmed by many subsequent workers These particular findings cannot be explained in terms of the classical theory of blood coagulation Nolf s work is therefore of the greatest importance but unfortunately his views were often obscurely expressed and the relevant experiments difficult to extricate from his writings The development of his views may be illustrated by quotations from his conclusions which have been translated as follows

Nolf (1908) 'The previous researches of the author have led him to consider the coagulation of the blood as the result of a mutual precipitation of three protein substances of the plasma thrombozyme thrombogen and fibrinogen Thrombozyme is secreted by the leucocytes and endothelial cells' The majority of the tissue cells do not contain thrombozyme They only contain substances which without being necessary for coagulation accelerate the process considerably These substances are said to be thromboplastic Nolf s thrombozyme is equivalent to the prothrombin of other workers and he postulates the existence of another substance in the plasma thrombogen which is necessary for clotting

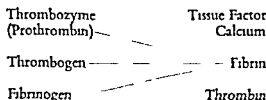
Nolf (1928) The accumulated evidence leads to the belief that Hammarsten s fibrinogen is a real constituent of normal plasma But Nolf has shown that it is incapable of uniting with thrombozyme (prothrombin) as long as it is in the presence of this substance in isolation For the union to be possible a third substance throm-

bogen is necessary This is present in excess in plasma but has not been isolated The formation of fibrin is due to the union into a single complex of thrombozyme thrombogen and fibrinogen' When mammalian plasma is treated with a gelatinous precipitate of $Ca_3(PO_4)_2$ or $Al(OH)_3$ the main part of the thrombozyme is removed leaving behind the fibrinogen and proteins of hepatic origin

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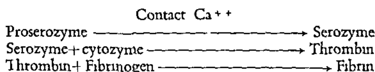
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most cells by centrifuging in paraffin lined containers clotted very slowly on the addition of calcium and after coagulation had occurred the serum contained much prothrombin (serozyme) On the addition of thromboplastin (cytozyme) the serum formed thrombin more rapidly than the original plasma (Bordet and Delange 1912 Bordet 1920)

Bordet suggested that the plasma contained a substance proserozyme which was altered by contact with foreign surfaces and calcium to form serozyme which reacted with a tissue factor to form thrombin This theory may be expressed as follows



This theory postulates a pro-prothrombin (proserozyme) and has the major disadvantage that it involves a hypothetical substance whose existence cannot be proved by any in vitro experiments Bordet's work has therefore often not received the attention that it deserves because his theory has been remembered while his experiments were forgotten Bordet's experiments can also be explained by the development of an accelerator of blood coagulation on contact of the blood with glass and during coagulation This interpretation is probably more in agreement with recent experiments than the theory proposed by Bordet

MODERN DEVELOPMENTS IN BLOOD COAGULATION

Recent studies in blood coagulation have led to a change of the main focus of interest For many years study centred on the conversion of prothrombin to thrombin Work initiated by the observations of Nolf suggested that the mechanism of this conversion was complex factors other than tissue extracts or thromboplastin and calcium being involved These studies have led to an inquiry into the nature of thromboplastin and the origin of thromboplastic activity from blood constituents Today many workers are therefore studying the reactions which precede the conversion of prothrombin

to thrombin From this work it is clear that thromboplastin is formed in the blood by a series of reactions much hypothetical discussion about the nature of these reactions has resulted and many new coagulation factors have been born The confusion is now naturally considerable and it is becoming very difficult for the new reader to distinguish those factors which have earned respectability by surviving the attacks of many generations of research workers from upstarts whose expectation of life is limited

In this book emphasis will be laid on observations which have been confirmed by at least two groups of workers In this way it is hoped that the factors with the greatest probability of survival have been given prominence

CHAPTER II

THROMBIN FIBRINOGEN THE THROMBIN-FIBRINOGEN REACTION AND FIBRIN

The appearance of fibrin in plasma follows the action of thrombin on fibrinogen Thrombin is formed as the product of a complicated chain reaction the stages of which are not yet clear Logically the stages in thrombin formation should be described before discussing the properties and action of thrombin This logical sequence cannot be followed because the initial phases leading to thrombin formation can be studied only by observing their effect on fibrin formation An understanding of the thrombin-fibrinogen reaction must precede a study of *thrombin formation*

FIBRINOGEN

Fibrinogen may be defined as the plasma protein clotted by thrombin it is a globulin of molecular weight about 400 000–500 000 (Nanninga 1946 Edsall et al 1947) The molecule is 3-4 times larger than other plasma globulins it is elongated being about 700–1000 Å in length and 30–50 Å in width According to Wohlisch and Kiesgen (1936) fibrinogen belongs to the fibrillar group of proteins and Bailey Astbury and Rudall (1943) from X-ray diffraction studies believe that it belongs to the myosin-keratin family

Fibrinogen is precipitated from human plasma by ¼–⅓ saturation with ammonium sulphate The iso-electric point is at pH 5 5 (Nordbo 1927) Fibrinogen is destroyed by heating to 47 C

PREPARATION OF FIBRINOGEN

Fibrinogen is usually prepared from plasma by precipitation with salts alcohol or ether The methods used for its preparation have varied with the view of blood clotting held by experimental workers and the type of experiments for which the fibrinogen was used Some of the main methods are outlined in Table 1 Hammarsten (1879) who was primarily interested in fibrinogen used the fraction

TABLE 1

METHODS USED FOR THE PREPARATION OF FIBRINOGEN

Author	*Source of Fibrinogen*	*Method used for Precipitation*	*Number of Re-precipitations*
Hammarsten (1879)	Horse plasma	An equal volume of saturated NaCl	6–7
Mellanby (1909 and 1933)	Bird plasma	Plasma diluted 10 × and acidified to pH 5 3	1
Cekada (1926)	Cat plasma	½ saturation with NaCl	3
Milstone (1941a)	Oxalated human plasma	½ saturation with $(NH_4)_2SO_4$	10
Jaques (1943)	Oxalated human plasma	M phosphate buffer	3
Astrup (1944)	Oxalated ox plasma treated with $Ca_3(PO_4)_2$	½ saturation with $(NH_4)_2SO_4$	2
Keckwick et al. (1946)	Citrated human plasma	11 volumes per cent of ether at 0 to 0 5 C.	2
Owren (1947)	Oxalated Seitz filtered ox plasma treated with $Ca_3(PO_4)_2$	½ saturation with $(NH_4)_2SO_4$	2
Cohn (1950)	Human blood collected into a non-wettable vessel and calcium removed by ion exchange resin Treated with $BaSO_4$	6 volumes per cent of ethyl alcohol at −2 C. and pH 6 8	0

precipitated by saturated sodium chloride and freed this component from other plasma proteins by repeated precipitations Mellanby (1909 and 1930) who studied the formation and action of thrombin used the fraction of plasma proteins precipitated by dilution and acidification to pH 5 3 Some workers (Owren 1947 Cohn 1950) have removed prothrombin from the plasma before precipitating the fibrinogen The species of animal used has varied as has also the method for making the precipitation

The purity of products used as fibrinogen solutions has varied considerably Two main criteria can be applied to these preparations Since fibrinogen is defined as the protein clotted by thrombin the purity of a fibrinogen solution can be assessed by determining the amount of protein which remains after the fibrinogen is removed by coagulation with thrombin Using this criterion Avery and

Monroe (1948) showed that the fibrinogen prepared by Jaques' (1943) phosphate buffer method was much purer than that prepared by precipitation with ammonium sulphate or sodium chloride The substance prepared by ether precipitation is also relatively pure (Keckwick et al 1955) The second method of assessing the purity of a fibrinogen preparation is to determine what other substances can be demonstrated Thus fibrinogen made by Mellanby s method contains Factor V, prothrombin, plasminogen and the antihaemophilic globulin in addition to fibrinogen Fibrinogen prepared by any of the known methods still contains the antihaemophilic globulin and usually also plasminogen This second method of assessing the purity of fibrinogen is in practice the most useful For any particular experiment care can be taken to exclude factors of known importance For example in experiments designed to measure the amount of prothrombin present in a sample of defibrinated plasma it is important that the fibrinogen used as an indicator of thrombin formation should be free from prothrombin

We have found that the preparation of fibrinogen used by Jaques is most reliable and the technique is described in the appendix

THE MEASUREMENT OF FIBRINOGEN

The determination of fibrinogen presents no difficulties if it is assumed that it is converted quantitatively into fibrin by thrombin The fibrinogen is removed from the solution to be tested by coagulation with thrombin The fibrin is carefully collected on a glass rod and washed to remove other proteins that may adhere to it The amount of fibrin recovered can then be estimated by a variety of methods one of which is given in the appendix

THROMBIN

Thrombin may be defined as the active coagulant of fibrinogen which appears in plasma during its coagulation From its precipitation by ammonium sulphate Astrup and Darling (1941) concluded that thrombin belongs to the albumin group of proteins Lamy and Waugh 1953 estimate the molecular weight as 45 000 Other active preparations were said to have a molecular weight of 75 000 (Astrup 1944) Electrophoresis shows three components with isoelectric points 3 6 4 1 and 4 7 (Seegers 1950) in citrate activated thrombin (see Chapter IV) Thrombin is precipitated between pH

5 1 and 3 4 and is destroyed by strong acids and alkalies and by heating to 60 C The purified substance contains no phosphorus (Chargaff Ziff and Cohen 1940 Astrup and Darling 1941) or calcium (Ferguson 1937)

PREPARATION OF THROMBIN

Thrombin is formed from prothrombin in plasma by the action of thromboplastin and calcium Schmidt (1892) precipitated it from fresh serum with alcohol Mellanby (1933) made a globulin fraction of plasma by dilution and acidification to pH 5 3 from which on standing the prothrombin spontaneously generated thrombin Astrup and Darling (1941) prepared a globulin fraction of plasma by the same method and thrombin was formed from it by adding beef lung thromboplastin and calcium chloride The thrombin was then precipitated with acetone and extracted from the precipitate with 0 9 per cent saline. Seegers et al (1938) used a similar method with the exception that prothrombin was used instead of the crude globulin fraction.

Highly purified thrombin has been prepared from isolated prothrombin by Seegers et al (1950) by the action of sodium citrate Using this method no extraneous clotting factors are added and the produce must therefore be said to be pure This purified product when tested electrophoretically was found to consist of three components Thus there is some doubt about the chemical purity of even the best preparations of thrombin

Relatively crude preparations made from the globulin fraction of stored blood-bank plasma by recalcification and acetone precipitation have as much as 50 units of thrombin per mg of dried substance By more elaborate methods of purification greater activity (950 units per mg) can be achieved In practice much of the thrombin is lost in purification and for most purposes solutions of thrombin containing 1-20 units per ml are usually sufficient The preparation of a highly purified substance is not necessary for many experiments A simple and effective method of preparation is given in the appendix

THE THROMBIN-FIBRINOGEN REACTION

The most important property of both thrombin and fibrinogen is their reaction together to form fibrin With the study of this

reaction which is apparently the simplest of the whole chain of reactions leading to clotting the main difficulty of all quantitative work on blood coagulation is revealed Using crude or partially purified biological materials it is not possible to obtain exactly reproducible results With carefully standardized conditions the results of comparable experiments will be similar but seldom identical

Two main methods have been used to follow the thrombin-fibrinogen reaction In the first the increasing turbidity of the solution containing fibrinogen and thrombin is followed photometrically (Nygaard 1941) This method probably measures the speed of polymerization of fibrinogen molecules which leads to coagulation when the network of polymerized molecules is sufficiently dense to give the semblance of solidity By this method coagulation is an incident in the reaction and the turbidity of the mixture increases after coagulation as usually recognized is complete

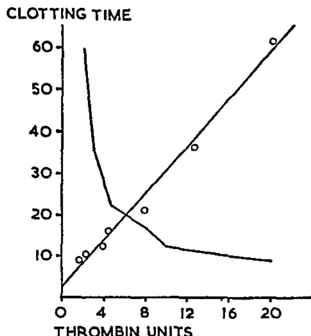


Fig 1 A number of dilutions of thrombin topical (Roche) were made and 0 1 ml of each dilution was added to 0 4 ml fibrinogen and the clotting time in seconds was recorded The results of this test are shown on the hyperbolic curve The straight line graph shows the clotting time plotted against the reciprocal of the thrombin concentration

As Lein (1948) has emphasized coagulation is an arbitrary stage in the reaction between thrombin and fibrinogen and a study of coagulation alone will give little idea of the type of reaction in progress From the practical point of view, it is the formation of a blood clot which is important and the majority of workers have followed the thrombin-fibrinogen reaction by recording the time taken for fibrinogen to clot with different concentrations of thrombin When the clotting times are related to the concentration of thrombin a hyperbolic curve such as that shown in Fig 1 is obtained. This curve can usually be converted to an approximate straight line by plotting the reciprocal of the concentration of thrombin against the clotting time This straight line passes very near to but not quite through the origin because however strong the concentration of thrombin the fibrinogen takes some time to clot This straight line relationship probably has no deep theoretical significance but is important because it forms the basis of the two-stage methods for the estimation of prothrombin Astrup and Darling (1941) have shown that impure preparations of fibrinogen do not show this relation and they should not be used for the two-stage prothrombin test

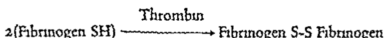
THE NATURE OF THE THROMBIN-FIBRINOGEN REACTION

Schmidt (1892) believed that fibrinogen was converted to fibrin enzymatically by thrombin That this view is correct is suggested by the fact that thrombin will convert many times its weight of fibrinogen to fibrin (Eagle 1935b Seegers et al 1945 Morrison 1947) Some doubts on the enzyme nature of thrombin arose from the fact that much of the thrombin added to fibrinogen disappears during coagulation This disappearance has now been shown to be due to the adsorbtion of thrombin by fibrin and therefore cannot be used as evidence against the enzyme nature of thrombin action (Klein and Seegers 1950)

From a study of the thrombin-fibrinogen reaction in pure solutions Lein (1948) Ferry (1948) and Steiner and Laki (1951) have suggested that the change from fibrinogen to fibrin is due to the polymerization of individual fibrinogen molecules Laki and Mommaerts (1945) suggested that the clotting of fibrinogen takes place in two stages In the first the thrombin acts on fibrinogen and in the second the altered fibrinogen molecules aggregate At pH 5 1 the first stage takes place but clotting does not occur unless the solution

is neutralized Laki and Mihalyi (1949) found that iodination of the tyrosine molecules in fibrinogen also inhibited the aggregation phase of clotting

The chemical alteration in fibrinogen which occurs during clotting is not fully worked out but it has been suggested by Baumberger (1941) that the SH groups present in fibrinogen may be oxidized by thrombin to form an S-S linkage



This supposition is supported by observations of Lorand (1954) on the structure of fibrin (see later)

Lyons (1945a and b) has also supported the two-stage hypothesis for the thrombin-fibrinogen reaction Lyons' hypothesis involves two types of thrombin and fibrinogen In the first stage fibrinogen A is converted to fibrinogen B by thrombin A In the second stage fibrinogen B is converted to fibrin by thrombin B At the moment this view is not substantiated and has the disadvantage of proposing the existence of four hypothetical factors whose reality cannot easily be proved

From the work of Bailey Bettelheim, Lorand and Middlebrook (1951) it appears that the action of thrombin on fibrinogen leads to the appearance of terminal glycine molecules which are not found in unaltered fibrinogen Lorand (1954) has shown that the appearance of these terminal glycine molecules follows the splitting off from fibrinogen of a fibrinopeptide of molecular weight 4000-8000 The peptide is highly acidic and its removal from fibrinogen leads to a loss of negative charges which might predispose to polymerization and fibrin formation

Sherry Troll and *Wachman* (1954) showed that thrombin can split a synthetic substrate p-toluene-sulphonyl-arginine-methyl ester with the liberation of methyl alcohol Lorand (1954) suggests that thrombin probably splits arginylglycine bonds

This work confirms the probable enzyme action of thrombin and *it is suggested that fibrin formation involves three stages first acidic* groups are removed then the molecules polymerize and finally evidence from the structure of natural clots suggest that in physiological fibrin formation the fibrin is strengthened by other possibly S-S linkages under the influence of calcium and some substance present in blood but lacking from purified solutions of fibrinogen

and thrombin (Lorand 1954 see also section on the structure of fibrin) According to Shulman (1953) the blood factor is in the albumin fraction of bovine serum

THE CONDITIONS WHICH INFLUENCE THE THROMBIN-FIBRINOGEN REACTION

The thrombin-fibrinogen reaction can be studied using purified fibrinogen or whole plasma as the substrate When whole plasma is used substances other than fibrinogen may have a great effect on the clotting time On the other hand if purified solutions of fibrinogen are used the observed reaction between thrombin and fibrinogen may bear little resemblance to that occurring in plasma

Using purified solutions many factors may alter the speed with which thrombin and fibrinogen react Many solutions of fibrinogen deposit a white sediment on standing in the cold This substance readily redissolves on warming and has been called soluble fibrin by Hammarsten (1879) and profibrin by Apitz (1938) It reacts much more rapidly to thrombin than does the supernatant solution (Owren 1947) It has been suggested that this form of fibrinogen is an intermediate product in the polymerization of fibrinogen to fibrin (Lein 1948) If different preparations of fibrinogen contain a variable amount of this substance their reaction to thrombin will naturally be variable Owren (1947) has suggested that the profibrin should be removed by centrifuging in the cold before the fibrinogen is used

Astrup (1944 1947) and Owren (1947) have shown that many other factors may influence the speed of the reaction between thrombin and fibrinogen Owren found that the concentration of fibrinogen is important particularly with dilute thrombin solutions the optimum being at about 0 1 gm. per cent Astrup and Owren have both demonstrated the importance of the concentration of neutral salts in the solution an excess leading to delayed clotting Alterations in pH beyond the range 6 5 to 7 5 are also important the optimum temperature is 37° C Fibrinogen prepared from different species behaves differently to the same thrombin solution The clotting time is affected by the presence of other proteins

In plasma many of the factors which influence the thrombin-fibrinogen reaction in pure solutions are automatically controlled The salt concentration and pH do not vary greatly from one sample of plasma to another The concentration of fibrinogen is fairly con-

TABLE 2

The clotting times in seconds of samples of normal and pathological plasma on the add tion of thrombin

Source of patient s plasma	*Normal*	*Patient*
Jaundiced patient	25 5	49
	8 5	19 5
	17 5	26 5
	11	18
	18 5	39
Infant	14	29
	11 5	23
	14	18
	11 5	21
	12	16
	16	40
	10	26 5
	9	23
	10 5	57
	21 5	60
	25	39

TABLE 3

The thrombin clotting times of pla ma with and without the addition of M/40 CaCl

Substrate	*Clotting time in seconds on the addition of thrombin and saline*	*Clotting t me in seconds of the addition of thrombin and $CaCl_2$*
Plasma from patients with liver dysfunction	19	9 5
	14	9 5
	11	9
	17	9
	16	7
	15	11
	12	8
Plasma from newborn infants	19	6
	31	12
	48	13
	32	16
	20	12
	33	11
	29	19
Normal plasma	14	9 5
Normal plasma adsorbed with an excess of $BaSO_4$	24	11

stant in normal plasma though the level is usually greater than the optimum for the thrombin-fibrinogen reaction In consequence some authors have preferred to follow the thrombin-fibrinogen reaction in plasma rather than in purified solutions A difficulty in the use of plasma lies in the fact that different samples of plasma may vary in the speed with which they clot on the addition of thrombin and important but unknown factors will complicate the results (see Table 2) For example an infant s plasma nearly always has a slower clotting time than an adult s plasma on the addition of thrombin (Biggs 1951)

When the fibrinogen from plasma is precipitated by 33 per cent saturation with ammonium sulphate the precipitate dissolved in saline and the solution dialysed it is found that this fibrinogen has a longer clotting time on the addition of thrombin than has the original plasma When plasma is treated with a large amount of $BaSO_4$ and the precipitate removed by centrifuging the supernatant plasma has a longer clotting time with thrombin than has the original plasma In plasma samples with a relatively long clotting time on the addition of thrombin the clotting time may be shortened by the presence of calcium Similarly after adsorbtion with $BaSO_4$ the clotting time may be shortened by $CaCl_2$ (Table 3) Ware Fahey and Seegers (1948) have shown that platelet extracts accelerate the thrombin-fibrinogen reaction

It is clear that very little is known about the thrombin-fibrinogen reaction in plasma. The existence of factors which accelerate the reaction is not certain but the possibility of their existence should be borne in mind if only because it is very difficult to distinguish between factors which accelerate thrombin formation and those which accelerate the thrombin-fibrinogen reaction

In plasma it is sometimes found that some of the fibrinogen may be clotted non-specifically by substances other than thrombin For example Eagle and Harris (1937) have shown that plasma is clotted by papain Chargaff and Bendich (1943) and Chargaff and Ziff (1941) have shown that other substances such as Chloramine-T ninhydrin potassium 1 4-naphthoquinone-2-sulphonate can clot fibrinogen The majority of these coagulants are oxidizing agents and from this fact Chargaff (1945) has suggested that oxidation may occur during normal coagulation Cummine and Lyons (1948) found that only certain plasma samples were clotted with naphthoquinone and held that the coagulation was due to the presence of

fibrinogen B in the solution They supposed that this coagulation was due to the oxidation by the naphthoquinone of sulph-hydryl groups present in the fibrinogen molecule leading to the formation of di-sulphide linkages between the fibrinogen molecules and the formation of a lattice of fibrin Since fibrinogen is not coagulated by naphthoquinone in all plasma samples it is suggested that fibrinogen containing these available sulph-hydryl groups is not normally present in the blood stream but is the product of a reaction between normal fibrinogen termed by them 'fibrinogen A and a component of thrombin known as thrombin A The effect of this reaction is to expose sulph-hydryl groups which are normally blocked in some way The exposure of these groups results in the formation of fibrinogen B which is then capable of being coagulated by oxidizing agents such as ninhydrin and naphthoquinone The presence of fibrinogen B which can be demonstrated according to Lyons by the coagulant effect of naphthoquinone on oxalated blood indicates that some activation of thrombin A has occurred in vivo this activation being the result of some thrombotic or pre-thrombotic state the demonstration of which is of considerable clinical interest Though the coagulation of fibrinogen by naphthoquinone undoubtedly occurs in certain cases the interpretation placed on this phenomenon by Lyons and the clinical significance attributed by him to it now appear to be in considerable doubt

THE MEASUREMENT OF THROMBIN

Thrombin is usually measured in units which are defined by the speed of clotting of fibrinogen There are various definitions which have been used (see Table 4) The variety of factors which may influence the reaction means that the conditions under which coagulation occurs must be very rigidly controlled Owren (1947) therefore defines his thrombin unit very carefully He says

One thrombin unit has been defined in this study as equal to the amount of thrombin which in a volume of 1 c c containing 0 1 per cent of fresh pro-fibrin-free human fibrinogen at a temperature of 37 C and pH 7 3 and sodium chloride concentration of 0 154 M causes coagulation in 15 seconds

Having once made this definition a calibration curve of the concentrations of thrombin and their corresponding clotting times can be made and from this unknown solutions can be compared with known solutions in terms of thrombin units

TABLE 4

THE CRITERIA USED BY VARIOUS AUTHORS TO DEFINE A UNIT OF THROMBIN

Author	*Substrate*	*Conditions specified*	*Clotting time equivalent to 1 thrombin unit*
Mellanby (1917)	1 c.c. of fibrinogen	Temperature 37° C.	40 minutes
Warner, Brinkhous and Smith (1936) (thrombin topical Roche)	1 c.c. of fibrinogen containing 0.08–0.1 gm. per cent	Temperature 26–28° C. pH 7.1–7.3	15 seconds
Lenggenhager (1940)	1 c.c. citrated plasma + 1 drop 5 per cent $CaCl_2$	Temperature 20° C.	60 seconds
Astrup and Darling (1941)	*1 c.c. oxalated plasma*	*Temperature 37° C.*	*30 seconds*
Keckwick, Mackay and Record (1946)	1 c.c. of fibrinogen containing 0.1–0.2 gm. per cent	Temperature 37° C.	15 seconds
Owren (1947)	1 c.c. of fibrinogen containing 0.1 gm. per cent and free from pro-fibrin	Temperature 37° C. pH 7.3 NaCl 0.154M	15 seconds

This absolute definition of a thrombin unit enables thrombin solutions of constant strength to be prepared but in practice the errors involved in determining the unit in different laboratories will be large. This difficulty can be eliminated by depositing a standard preparation of known activity in a reference laboratory. This has been done for the preparation described by Warner, Brinkhous and Smith (1936). Even in one laboratory different batches of fibrinogen prepared in the same way vary in their reaction to thrombin (Table 5). Fibrinogen samples prepared by different methods are even more variable in their reaction. In practice the variable reaction of different fibrinogen preparations makes it impossible to devise a universal thrombin unit which will be applicable in all laboratories without frequent reference to a standard. Fortunately it is usually not necessary to have a universal unit: all that is required is that the results of a single experiment involving thrombin units shall have a constant criterion for the definition of the unit. It is not difficult to ensure a constant unit for so circumscribed a problem.

TABLE 5

In this experiment 0 1 ml amounts of the different thrombin concentrations were added to 0 4 ml amounts of each of the fibrinogen preparations

Samples of fibrinogen made by the phosphate buffer method tested with the same solutions of thrombin				
Concentration of thrombin in units/ml	*Clotting time in seconds of different fibrinogen samples*			
	1	2	3	4
20	9	11	7	9
15	10	12	8	10
10	13	17	10	12
8	16	20	12	11
5	22	31	18	25
3	36	46	25	36
2	54	70	42	61
1	9-	164	77	178

FIBRIN

Fibrin is a substance formed from fibrinogen after the action of thrombin Fibrin appears to have the same gross chemical constituents as fibrinogen though Lorand and Middlebrook (1952) have shown that the terminal groups of bovine fibrinogen are tyrosine and glutamic acid while those of fibrin are tyrosine and glycine When thrombin is added to fibrinogen the formation of fibrin can be studied with the electron microscope (Van Zandt et al 1947 Porter et al 1949) Within 15 seconds of mixing end to end linkage occurs between particles of the order of size of fibrinogen molecules Later these fibrils are aggregated into bundles which have regular cross striations of different density (see frontispiece)

Ferry and Morrison (1947) have made a detailed study of the factors which influence the structure of fibrin clots They have found that two types of clot can be differentiated one is transparent and very friable and the other opaque non-friable and with a tendency to retract and expel the solvent The character of the clots depends on the conditions under which clotting occurs High fibrinogen concentration favours opacity Increase in pH and ionic concentration gives rise to transparent clots The factors which encourage the formation of transparent clots are those which delay

clotting and reduce the forces of attraction between fibrinogen molecules From their work Ferry (1948) suggests that fibrinogen molecules are held together by both primary and secondary linkages The primary linkages it is suggested, are catalysed by thrombin whereas the secondary forces of attraction account for the effect of ionic strength and pH etc on the structure of the resulting clot

Lorand (1950) has studied the solubility of fibrin clots in 30 per cent urea He finds that fibrinogen clotted in plasma is insoluble in urea whereas clots formed in purified solutions of thrombin and fibrinogen are soluble Lorand holds the view that the clots that form in purified fibrinogen solutions are held together by secondary linkages only whereas in those formed in plasma the linkages are reinforced by primary bonds The solution of fibrin in urea reclots spontaneously if the urea is removed by dialysis This process can be repeated many times and by using large volumes of solvent any adhering thrombin should be eliminated Since the fibrin always clots on removal of the urea it seems probable that thrombin induces some permanent change in the fibrinogen molecule which predisposes to polymerization Lorand has found that the failure of clots formed in plasma to dissolve in urea depends on the presence of a factor present in normal serum and on the presence of calcium The insoluble plasma clots are soluble in a mixture of urea and thioglycollic acid suggesting that S-S bonds may be involved in natural clotting (Lorand 1954)

SUMMARY TO CHAPTER II

The clotting of fibrinogen with thrombin the final stage of coagulation and the only directly observable phenomenon follows a chain of earlier reactions information about which is all indirect

Various procedures for the purification of thrombin and fibrinogen are described As usually prepared neither of these substances are freed from all other proteins

When thrombin clots purified fibrinogen the clotting time of the fibrinogen is inversely related to the thrombin concentration Many factors affect the speed of clotting A unit of thrombin activity can be defined in terms of fibrinogen or plasma clotting times the determination of such units will vary very much from one laboratory to another No attempt has been made to establish an absolute thrombin unit for experiments described in this book.

The clotting of fibrinogen is due to the polymerization of altered fibrinogen molecules It is thought that thrombin removes acidic groups from the fibrinogen which then polymerizes In blood clots the union of the polymerized fibrin molecules is probably strengthened by S-S linkages

PROTHROMBIN

In the classical theory prothrombin was proposed as a hypothetical precursor of thrombin Evidence has now confirmed the probable existence of prothrombin and two lines of research have produced much interesting information Prothrombin has been isolated from plasma and the properties of the isolated substance have been studied In addition methods for the measurement of prothrombin activity have been devised and these methods have been used to study haemorrhagic disorders in patients

THE PREPARATION AND PROPERTIES OF PROTHROMBIN

Prothrombin can be separated from some of the other plasma constituents by differential precipitation with salts such as ammonium sulphate, or by changing the protein solubility by altering pH and ionic concentration or by adsorbtion on to inorganic precipitates e g $Ca_3(PO_4)_2$ (Bordet and Delange 1914) $Mg(OH)_2$ (Fuchs 1929) $Al(OH)_3$ (Quick 1935b) and $BaSO_4$ (Dale and Walpole 1916) Some of the methods that have been used are summarized in Table 6 A simple method using $Al(OH)_3$ or $BaSO_4$ as adsorbant is described in the appendix

The properties of these prothrombin products are variable The substances prepared by acid or salt precipitation readily generate thrombin in the presence of tissue thromboplastin and calcium. If different dilutions of these preparations are used it is found that the amount of thrombin formed is proportional to the amount of substance present (Eagle 1935 Herbert 1940)

The substances made by adsorbtion do not react well with thromboplastin and calcium Thrombin is formed very slowly (Ware and Seegers 1948a, Owren 1947 Fantl and Nance 1948) or not at all (Nolf 1945) At first this nonreactivity was thought to be due to denaturation but it was found that rapid thrombin formation could be restored by adding to the isolated prothrombin the plasma from which the prothrombin had been removed (Owren 1947

TABLE 6

Methods f r the preparation of prothrombin

A th r	*Plasma used*	*Method for removal of fibrinogen*	*Method for the isolation of the globulin fraction*	*Method for the adsorption and purification of prothrombin*
Mellanby (1909 and 1930)	Bird plasma	None	Plasma diluted 10 × and acidified to pH 5 3	None
Bordet and Delange (1914)	Bird and rabbit plasma	None	None	$Ca_3(PO_4)_2$ Prothrombin released with CO_2
Howell (1914a)	Oxalated cat plasma	None	Whole plasma precipitated with an equal volume of acetone	None
Cekada (19 6)	Oxalated cat plasma	Plasma heated to 56 C	Whole plasma precipitated with acetone and on solution prothrombin precipitated by 35 50 per cent saturation with $(NH_4)_2SO_4$	None
Mellanby (1930)	Oxalated cat plasma	None	Plasma diluted 10 × and acidified to pH 5 3 Solution treated with CO_2 and reprecipitated	None
Seegers (1940)	Oxalated ox plasma	None	Plasma diluted 10 × and acidified to pH 5 3	Dissolved globulins treated with $Mg(OH)_2$ Prothrombin released with CO_2
Nolf (1945)	Human Plasma	None	None	$Ca_3(PO_4)_2$ Prothrombin released with phosphate buffer at pH 8
Seegers et al (1945) Ware and Seegers (1948c)	Oxalated ox plasma	None	Plasma diluted 15 × and acidified to pH 5 1	Dissolved globulins treated $Mg(OH)_2$ Prothrombin released with CO_2 and reprecipitated by 65 per cent saturation with $(NH_4)_2SO_4$
Owren (1947)	Oxalated ox and human plasma	Plasma heated to 56 C	Plasma diluted 16 × and acidified to pH 5 5	Dissolved globulins adsorbed with $Mg(OH)_2$ and prothrombin released with CO_2 Prothrombin reprecipitated at pH 5 3

Table 6 (cont)

Author	*Plasma used*	*Method for removal of fibrinogen*	*Method for the isolation of the globulin fraction*	*Method for the adsorption and purification of prothrombin*
Milstone (1945)	Citrated ox plasma	Heated to 51 C for 5 minutes	Plasma diluted to x and acidified to pH 5 3	Dissolved globulin adsorbed with $Mg(OH)_2$ and prothrombin released with a mixture of sodium acetate and acetic acid at pH 5 5-4 5
Fantl and Nance (1945)	Oxalated rabbit or human plasma	None	None	Plasma adsorbed with $BaSO_4$ and the prothrombin released with phosphate buffer at pH 8

Nolf 1945 Ware and Seegers 1948a Fantl and Nance 1948) This addition also causes an increase in the total amount of thrombin formed (Fig 2) This unreactive prothrombin appears to be the true precursor of thrombin because it will form thrombin very slowly in the presence of sodium citrate alone (Seegers McClaughry and Fahey 1950) and it must therefore contain all that is essential for thrombin formation When varying concentrations of this prothrombin are tested it is found that the amount of thrombin formed depends on the amount of prothrombin present (Owren 1947)

From these experiments it appears that prothrombin is the essential precursor of thrombin but that another substance which is not adsorbed by inorganic precipitates accelerates thrombin formation This substance will be called Factor V because Owren (1947) used this name and he made the most careful study of its properties Recently Owren (1950) has proposed a new name for Factor V pro-accelerin this name will not be used because the experimental proof that Factor V is a precursor substance is not yet sufficient (see Chapter V)

The prothrombin prepared by salt or acid precipitation constitutes about 1-3 gm per 100 ml plasma and is known to contain fibrinogen Factor V the antihaemophilic globulin and plasminogen but no antithrombin By no criteria can these products be said to be pure

To prepare prothrombin by adsorbtion one of the many available agents must be selected. Ware and Seegers (1948c) prefer $Mg(OH)_2$,

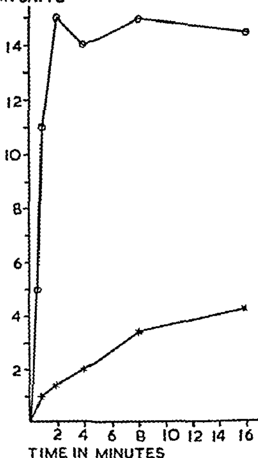


Fig. 2. The two-stage test carried out on mixtures containing prothrombin, brain emulsion and calcium chloride x—x and prothrombin, Factor V, brain emulsion and calcium chloride o—o

which is said to adsorb both prothrombin and Factor V. These two constituents are separated by differential precipitation with ammonium sulphate. In this laboratory it has been found that $Mg(OH)_2$ adsorbs prothrombin much more readily than Factor V and a separation can be achieved by adsorbtion alone. Owren (1947) tested $BaSO_4$, $Al(OH)_3$, Ca_3PO_4 and $Mg(OH)_2$. He found that all of

these substances adsorb some Factor V and that $BaSO_4$ adsorbs more Factor V than the other preparations It seems probable that details of preparation have a marked effect on the properties of the adsorbant. The selection of a suitable adsorbant is largely a question of trial and error in a particular laboratory In our experience $Al(OH)_3$ prepared by the method of Bertho and Grassman (1938) and $BaSO_4$ prepared as described in appendix III A 9 have given the most reproduceable results

Prothrombin prepared by adsorbtion constitutes about 16 5 mg per 100 ml plasma and has an activity of up to 1000 units per mg of dried protein The prothrombin prepared by adsorbtion and differential precipitation with $(NH_4)_2SO_4$ has greater activity Seegers McClaughry and Fahey (1950) and Seegers and Andrews (1952) have prepared prothrombin with an activity of 1400–2000 units per mg of dried protein or 23 000–30 000 units per mg of tyrosine the tyrosine constituting 4.58 per cent of the protein This protein is present in whole plasma in a concentration of 20 mg per cent or 300 units per ml Unfortunately preparations made by the lyophil drying method lose much of their activity on storage

Dried prothrombin contains sulphur 3 33 per cent of tryptophane 4 58 per cent of tyrosine and 6 per cent of carbohydrate (Seegers Loomis and Vandebelt 1945 Seegers McClaughry and Fahey 1950) The molecular weight is 63 000 (Lamy and Waugh 1953) Laki et al. (1954) analysed some purified prothrombin and found 18 amino acids the sulphur being present as cystine and methionine. The optimum pH for activation is 7 – and prothrombin is insoluble between pH 3 9 and 5 6 Electrophoretic analysis of the prothrombin produced in Seegers laboratory showed one main component which constituted 87–95 per cent of the total The mobility corresponds approximately to α_1-globulin (Seegers 1955) In whole plasma prothrombin is precipitated at 50 per cent saturation with ammonium sulphate but from the protein adsorbed by $Mg(OH)_2$ it is precipitated between 50 and 65 per cent saturation

According to Owren (1947) prothrombin in solution is destroyed by heating to 60 C and inactivation starts at 40 C On the other hand Ware and Seegers (1948c) have heated neutral prothrombin solutions in distilled water for 53 C for two hours In this laboratory prothrombin prepared by $Al(OH)_3$ adsorption withstood heating to 56 C for seven hours (Fig 3) and boiling a finding which has been confirmed by Hjort (1955) This prothrombin preparation

also retained activity after precipitation with an equal volume of 10 per cent trichloroacetic acid and re-solution.

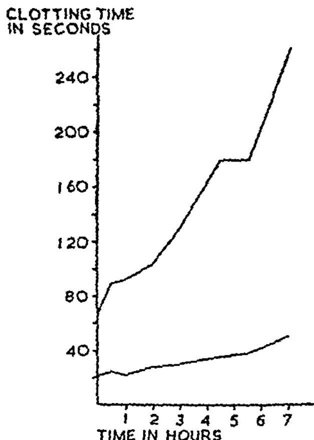


Fig 3 The one-stage clotting time of mixtures of prothrombin Factor V brain emulsion, fibrinogen and calcium chloride (lower curve) and prothrombin saline brain emulsion fibrinogen and calcium chloride (upper curve) The prothrombin preparation used was heated to 56 C for 7 hours and samples were tested at intervals

Most preparations of prothrombin are activated by boiled tissue extract and calcium alone and activation is usually complete by ½-2 hours the yield of thrombin is less than that in the presence of Factor V Preparations which have been heated to 56° C for 7 hours or boiled are similarly activated Since Factor V is destroyed by heating to 56 C for 15-30 minutes it must be concluded that prothrombin can be converted to thrombin by tissue extract and calcium alone The great heat stability of the prothrombin prepared by

adsorption is surprising It is possible that some substance adsorbed together with the prothrombin has a protective effect, or that in plasma prothrombin is associated with a heat labile protein

The functional purity of prothrombin preparations can be determined by testing for the presence of other known coagulation factors When prothrombin made by adsorbtion on to $Al(OH)_3$ is added to haemophilic plasma there is no significant shortening of the clotting time of the haemophilic plasma (Table 7) It appears that this preparation does not contain the antihaemophilic globulin and other tests demonstrate that it is also free from fibrinogen antithrombin and plasmin As was suggested earlier there are no ways of

TABLE 7

The effect of prothrombin prepared from normal and haemophilic plasma on the clotting time of haemophilic plasma

The fractions of plasma were reconstituted to their original volume

0 1 ml. of the substance tested was added to 0 1 ml of haemophilic plasma and the mixture recalcified with 0.1 ml of M/40 $CaCl_2$ The clotting times are recorded in minutes

Substance added to haemophilic plasma	*Experiment I*	*Experiment II*
Saline	6	9
Normal prothrombin	5	7½
Haemophilic prothrombin	5½	10
Fibrinogen fraction from normal plasma	2½	2

discovering whether prothrombin is entirely free from all other clotting factors Even the electrophoretic homogeneity is not conclusive evidence of purity because two components with similar electric charge cannot be differentiated if the molecules are of the same size and shape

THE ACTIVATION OF PROTHROMBIN BY SODIUM CITRATE

Much of this book is concerned directly or indirectly with the biological activation of prothrombin and the factors which influence

the reaction In this chapter the artificial reactions of purified prothombin are considered The observations of Seegers McClaughry and Fahey (1950) and Seegers (1955) on the activation of purified prothrombin with 25 per cent sodium citrate may have little bearing on the physiological process but the reaction has been studied in some detail and the results are of interest in themselves During citrate activation there is a lag period of four hours during which no thrombin appears thereafter the rate increases and complete conversion is achieved in twelve hours Electrophoresis during this reaction shows that during the lag phase the main prothrombin component changes and carbohydrate and protein derivatives soluble in 7 per cent trichloracetic acid are formed During this phase prothrombin disappears since the product cannot be activated by tissue extract and $CaCl_2$ As the period of incubation increases the one electrophoretic component becomes three components of differing electrophoretic mobility two of which have thrombin activity

When thrombin is formed in the presence of Factor V thromboplastin and $CaCl_2$ very little protein and carbohydrate material which is soluble in trichloracetic acid is liberated and the thrombin product has a molecular weight almost the same as that of prothrombin (Seegers 1955) This biothrombin can be converted to citrate thrombin by incubation with 25 per cent sodium citrate These results are very interesting They show that material with thrombin activity is formed by two apparently dissimilar reactions and that substances of differing molecular size have thrombin activity Whether or not the natural activation of prothrombin involves an inactive intermediate product is unknown but several hypotheses postulate such a product (Bordet 1920 Quick and Stefanini 1949) For the present there is no reason to assume that the citrate activation bears any relation to the physiological process though analysis of the type of thrombin formed in various pathological states might disclose different modes of activation Seegers (1955) distinguishes clearly between *citrate thrombin* and *biothrombin* according to the process of activation

THE MEASUREMENT OF PROTHROMBIN

The methods for the measurement of prothrombin activity when first proposed were all based on the classical theory of blood coagulation and designed to measure prothrombin in whole plasma Since

the classical theory is no longer adequate to explain all of the phenomena these methods are liable to give misleading results unless they are suitably modified and cautiously interpreted Although the methods were evolved to measure prothrombin in plasma they can be understood more easily when applied to isolated coagulation factors In this chapter the methods will be discussed only in relation to experiments with separated factors and in Chapters XI and XIV the application of the methods to plasma will be described

There are two main methods for the measurement of prothrombin the one-stage method first described by Quick (1935) and the two-stage method of Warner Brinkhous and Smith (1936) In the one-stage method tissue extract and calcium are added in optimum amounts to a solution containing prothrombin and fibrinogen and the clotting time of the mixture is recorded According to the classical theory the speed of thrombin formation depends on the concentration of prothrombin tissue extract and calcium if tissue extract and calcium are present in optimum concentrations then the speed of the reaction will depend only on the amount of prothrombin present and the clotting time of the mixture will give a measure of prothrombin

In the two-stage method thrombin is formed in a mixture of prothrombin tissue extract and calcium chloride At intervals samples are withdrawn from this incubation mixture and added to samples of fibrinogen solution the clotting times of which are recorded. The clotting times of the fibrinogen give a measure of the amount of thrombin present, and when the maximum thrombin concentration is reached it is supposed that all of the prothrombin is converted and that the thrombin level at this point gives a measure of the amount of prothrombin present

Both of these methods are useful and both of them require to be modified in experiments with isolated coagulation factors to include Factors V and VII Without these a maximum activation of prothrombin will not occur The two-stage method will be discussed first because although it is technically the more complicated of the two methods the interpretation of its results is easier

THE TWO-STAGE METHOD FOR THE MEASUREMENT OF PROTHROMBIN IN EXPERIMENTS WITH COAGULATION FACTORS

In this method the prothrombin preparation which contains factor VII is mixed with an excess of Factor V and to this mixture is

added tissue extract and calcium chloride Thrombin is formed in this mixture and at intervals samples are removed and added to a standard amount of fibrinogen the clotting times of which are recorded When this is done it is found that the clotting time of the fibrinogen shortens until a minimum is reached when an ap-

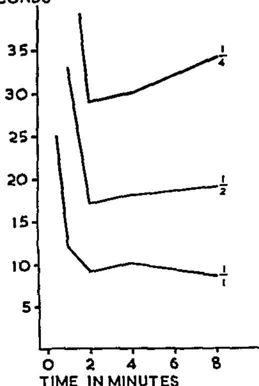


Fig 4 The two-stage test was carried out in a mixture of prothrombin Factor V brain emulsion and calcium chloride The test was repeated using the prothrombin preparation diluted 1/2 and 1/4 The clotting times of fibrinogen, to which samples from the incubation mixture had been added are plotted against the time at which the samples were removed

proximately constant clotting time is found (Fig 4) This constant clotting time occurs when all of the prothrombin has been converted to thrombin If this procedure is carried out with a number of dilutions of prothrombin and the minimum clotting times are plotted against the relative concentration of prothrombin calculated

from the dilution a hyperbolic curve such as that shown in Fig 5 is obtained Thus curve may be converted to a straight line by plotting the concentration of prothrombin as a reciprocal From Fig 5 it

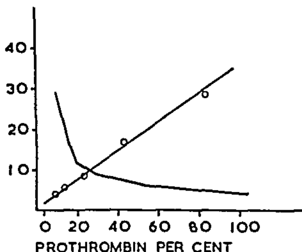


Fig 5 Various dilutions of prothrombin were tested by the two-stage method in the presence of Factor V brain emulsion and calcium chloride The minimum clotting time obtained with the dilutions was plotted against the relative percentage of prothrombin (hyperbolic curve) The straight line graph shows the percentage of prothrombin expressed as a reciprocal plotted against the clotting time

will be seen that this line passes very nearly through the origin Thus for practical purposes the relation between the clotting time and concentration of prothrombin may be written

$$\text{Clotting time} = \frac{K}{\text{Concentration prothrombin}}$$

Provided that the same solution of fibrinogen is used for any particular test the relative strengths of two prothrombin solutions can be compared This test is independent of the reactivity of the fibrinogen solution provided that the fibrinogen reacts to thrombin in the general way described in Chapter II The test is also independent of the tissue extract activity

Since this test is in fact measuring the amount of thrombin formed from an equivalent amount of prothrombin the clotting times illustrated in Fig 4 can be recorded in terms of the amount of thrombin formed by converting the clotting times to thrombin units from a thrombin dilution curve such as that shown in Fig 1 When this is done the curves of Fig 4 become those in Fig 6 This method of

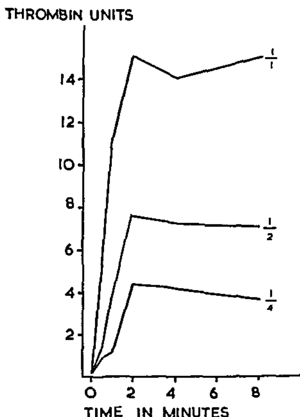


Fig 6 The results of the experiment illustrated in Fig 4 are shown. The clotting times have been converted to relative thrombin units using a dilution curve such as that shown in Fig 1

expressing results was used in Fig 2 and is particularly valuable in experiments designed to study the speed of thrombin formation

The two-stage method is simple to use and invaluable for studying the interactions of isolated coagulation factors As a routine method for the estimation of prothrombin in plasma the method must be

modified for reasons which will be discussed in greater detail in Chapter XI

THE ONE-STAGE METHOD

The one-stage method for the measurement of prothombin is apparently much simpler than the two-stage method but the interpretation of its results is more difficult

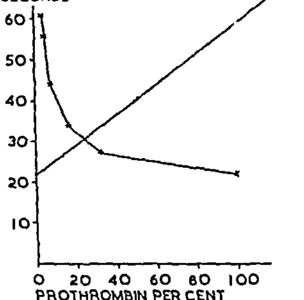


Fig 7 The one-stage clotting time was measured in mixtures of prothrombin, Factor V brain emulsion, fibrinogen and calcium chloride Various dilutions of prothrombin were tested The clotting times are plotted against the relative concentration of prothrombin (hyperbolic curve) The straight line shows the percentage of prothrombin expressed as a reciprocal plotted against the clotting time

In the one-stage method the solution containing prothrombin and Factor VII is mixed with Factor V and tissue extract and fibrinogen and calcium chloride are added to the mixture The clotting time of this mixture from the time of adding the calcium is recorded Where the two-stage method may require the measurement of 5 or 6 clotting times only one is made in the one-stage method

If this one-stage test is carried out with different dilutions of prothrombin a hyperbolic curve such as that shown in Fig 7 is obtained.

This curve can be converted to a straight line by plotting the reciprocal of the prothrombin concentration against the clotting time From Fig 7 it will be seen that the line does not pass through the origin but in this instance through a point at about twenty-one seconds The deviation of the line from zero is probably related to the initial delay in thrombin formation and depends to some extent on the activity of the tissue extract preparation The relation between the clotting time and the concentration of prothrombin must therefore be written

$$\text{Clotting time} = a + \frac{k}{\text{Concentration prothrombin}}$$

Thus no simple proportional calculation can give the relative concentrations of prothrombin in two preparations The clotting times must be converted to prothrombin concentration from a curve such as that shown in Fig 7 The exact shape of this curve will be affected both by the reactivity of the fibrinogen and the strength of the tissue extract A new curve must be made for each experiment

The clotting time by the one-stage method represents the resultant effects of the amount of thrombin formed the speed of its formation and the reactivity of the fibrinogen Unless the experiment is very carefully controlled it is difficult to distinguish the three factors with certainty In experiments with isolated coagulation factors the two-stage method is usually preferable to the one-stage method In routine tests on plasma the one-stage test is usually to be preferred not because the results give a more reliable measure of prothrombin but for the practical reason that the results of the test have been correlated with the clinical condition of the patient and the test can be interpreted very reliably in terms of the degree of coagulation abnormality and the probable tendency of the patient to bleed

UNITS OF PROTHROMBIN

The amount of prothrombin present in a preparation is sometimes measured in terms of the number of units of thrombin that can be formed from it under optimum conditions It is then said that a unit of prothrombin is that amount of prothrombin which is capable of forming 1 unit of thrombin Owren (1947) and Ware and Seegers (1948c etc) have used this terminology

DIVERGENT VIEWS ON THE NATURE OF PROTHROMBIN

In this chapter it has been assumed that prothrombin is a single substance which is quantitatively converted to thrombin The main deviations from this view have concerned the structure of prothrombin Is prothrombin a single substance or are there two components? Is the prothrombin in the plasma converted directly to thrombin or is it an inactive precursor which requires activation before it can be converted to thrombin?

Quick (1943) held that prothrombin was composed of two parts Prothrombins A and B Prothrombin A was a substance which deteriorated on storage and Prothrombin B was stable It has since been shown that Prothrombin A later known as the labile factor is synonymous with Factor V Quick (1947b c, d) then redefined his Prothrombins A and B in terms of the coagulation defects he observed in two patients These patients both had abnormal prothrombin times by the one-stage method and neither lacked Factor V The defect in the two patients was different and therefore Quick stated that there must be two parts of prothrombin Prothrombin A being deficient in one patient and B in the other Later (Quick and Stefanini 1949a) it was suggested that Prothrombin A is an inactive precursor of Prothrombin B Bordet (1920) also believed that the prothrombin in plasma was an inactive precursor substance (proserozyme) which was activated during clotting to a reactive form (serozyme) Lyons (1952) supports the view that prothrombin in plasma exists partly in a combined form. He suggests that some of the plasma prothrombin is combined with fibrinogen and that this combination is broken during the process of clotting The experimental evidence to support this hypothesis is not yet available Howell (1935) held that prothrombin was a complex of thrombin and heparin Recently this view of the nature of prothrombin has been revived by Wenckert and Nisson (1954 1955) who claim to have demonstrated an anticoagulant resembling heparin in fractions of plasma which contain prothrombin

Thus the divergence from the classical theory is seen to concern the nature of prothrombin in plasma In practice it would be extremely difficult to prove whether or not prothrombin underwent any reaction prior to its conversion to thrombin and thus the precursor prothrombin of Bordet and Quick is a hypothetical substance whose existence is as yet far from proved

SUMMARY TO CHAPTER III

Prothrombin is the one essential precursor of thrombin under favourable conditions prothrombin can be converted quantitatively to thrombin Thrombin can also be formed in large amounts by the addition of sodium citrate to prothrombin which contains only traces of other contaminating clotting factors

Prothrombin can be freed by fractionation procedures from fibrinogen antihaemophilic globulin plasminogen fibrinogen and antithrombin Most prothrombin preparations contain some other clotting factors

TISSUE EXTRACTS

It has long been known that tissue extracts accelerate blood coagulation though they do not clot oxalated blood or fibrinogen solutions The active principle is therefore a factor which accelerates the clotting of blood or plasma in the presence of calcium According to the classical theory these observations are interpreted to show that tissue extracts act by converting prothrombin to thrombin

THE NATURE OF THE TISSUE ACTIVATOR

Using this hypothesis many workers have tried to discover the nature of the tissue activator Schmidt (1892) recognized that alcoholic extracts of tissues accelerated clotting and that their activity resisted boiling Bordet and Delange (1913) thought that the active substance was a lipoid of the lecithin group Other workers (Howell 1912 McLean 1916 Gratia and Levene 1921) held that the lipoid was cephalin These workers used impure preparations and Fischer and Hecht (1934) showed that the activity of 'cephalin suspensions decreased with increasing purity Morawitz (1905) showed that aqueous extracts of tissues had greater activity than alcoholic extracts and Howell (1912) suggested that the active lipoid was normally associated with a protein The work of Feissly (1945a) also suggests that the tissue activator may have lipoid and protein components Chargaff and his co-workers (1944) have prepared a purified lipoprotein activator from lung From this work it is probable that the tissue factor is a lipoprotein in which the lipoid part is related to cephalin The active component must be very large because practically all the thromboplastic activity can be removed from ordinary tissue suspensions by centrifuging at 10 000 r p m and Chargaff s purified preparation can be sedimented by centrifuging at 25 000 r p m

THE PREPARATION OF TISSUE EXTRACTS

Extracts from tissues can be prepared by washing the fresh tissue free from blood, grinding up the residue with sand, and extracting

the ground tissue with saline Alternatively, the tissue may be dried with acetone and the dried tissue extracted with saline This last method is usually applied to brain tissue because it is very readily freed from large superficial blood vessels and it is easily reduced to a granular material in a mortar without sand

The preparations used by different workers have differed to a degree unusual even in blood coagulation work. Some use extracts of fresh tissue and some prefer it dried some use brain tissue and of those who prefer brain, some get it from rabbits, and some from human sources Some use extracts of placenta and some use lung Many workers use the venom of the Russell s viper One author prefers human milk to all other preparations This diversity of procedure makes it impossible to give a standard method for the preparation of tissue extracts one suggested method for preparing an extract from human brain tissue is given in the appendix

THE ACTIVITY OF PREPARATIONS FROM DIFFERENT TISSUES

Preparations from different tissues vary in their relative activity The most active and most widely used are extracts derived from brain lung and placenta The degree of activity can be assessed by the speed of clotting of normal plasma on the addition of the preparation and calcium From Table 8 it will be seen that there are large differences in activity

Qualitative difference in activity is less easily detected but that such a difference exists can be deducted from difficulties which have arisen in the use of the one-stage prothrombin time test If preparations all have the same mode of action but differ only in their degree of activity then the different preparations should react similarly to the factors which influence their activity One of these factors which has been most widely studied is the abnormality in the plasma of patients treated with dicoumarin In practice it is found that the lengthening of the clotting time of dicoumarin plasma is very different when tested with different tissue extracts When brain is used the lengthening in clotting time is greater than when Russell s viper venom is used (Biggs and Macfarlane 1949) Lung and brain do not give the same results From these observations it appears that the mode of action of the different preparations cannot be the same In a comparison of the actions of Russell s viper venom and brain

TABLE 8

The clotting time by the one-stage technique of a sample of normal plasma using various tissue extracts

The clotting time of a pathological plasma was tested by the one-stage technique using some of the different extracts and the percentage of prothrombin was read from appropriate dilution curves.

Type of tissue extract used	*Clotting time in seconds by the one-stage technique*	*Percentage of prothrombin recorded in one pathological plasma using different thromboplastins*
Fresh spleen extract	14	22
Fresh liver extract	28	15
Dried brain extract	27	5
Dried spleen extract	27	5
Dried liver extract	117	—
Dried lung extract	115	—
Dried platelet extract	55	20
Dried liver extract and lecithin	64	30
Dried lung extract and lecithin	76	—

Macfarlane et al (1941) showed that if plasma is freed from lipoid the activity of Russell s viper venom disappears An increase in the lipoid content of the plasma following a fatty meal leads to a shortening of the clotting time with Russell s viper venom (Fullterton and Anastasopoulos 1949) The activity of Russell s viper venom is therefore greatly affected by the lipoid content of the plasma The reaction of plasma to brain is unaffected by changes in plasma lipoid

SPECIES SPECIFICITY OF TISSUE EXTRACTS

The wide differences in activity of homologous tissue extracts suggests that there would be a marked species specificity of tissue extract action Trevan and Macfarlane (1936a) have shown that lung preparations from different species give varied coagulation times with the plasma samples from different animals (Table 9)

Quick (1942) recorded similar results using brain An interesting observation by Mann and Hurn (1952) suggests that the species specificity may be due to a specificity of serum co-factor rather than of tissue extract They found that incubation of the tissue extract with homologous serum removed the species specificity

TABLE 9

The clotting time by the one stage technique of plasma samples from different animals tested with lung extract from different animals (Trevan and Macfarlane 1936a)

Source of lung extract	*Clotting time in seconds by the one stage technique of the plasma from different animals*			
	Fowl	*Dog*	*Horse*	*Cow*
Fowl	22	72	255	—
Dog	1155	13	29	—
Horse	375	18	22	—
Cow	—	—	24	15

FACTORS WHICH INFLUENCE THE ACTIVITY OF TISSUE EXTRACTS

When human brain is added to normal oxalated or citrated plasma together with $CaCl_2$ the clotting time of the mixture is shorter than that of plasma to which $CaCl_2$ alone has been added The extent of this accelerating effect is altered by numerous factors and information about these can be obtained from experiments using fractions of normal plasma and pathological plasma samples Among the most important factors are temperature which is optimum at 37 C the concentration of tissue extract and the concentration of calcium

The Concentration of the Extract When plasma is mixed with increasing concentrations of extract, the clotting time of the mixture on recalcification shortens until a minimum is reached When the concentration of some preparations is increased above this optimum there is a lengthening of clotting time (Aggeler and Lucia 1938) Tocantins et al (1948) have shown that an inhibitory substance can be extracted from brain tissue it is possible that crude brain extracts contain both inhibitory and activating substances as suggested by Wright (1892) the inhibitory substance presumably being present in low concentration since its effect is readily removed by dilution

Calcium The activity of brain extract is markedly affected by the concentration of calcium There is a definite optimum range the clotting time being lengthened in the presence of concentrations above or below the optimum (Jaques and Dunlop 1945a and b) For most tissue extracts the optimum is about M/40 $CaCl_2$

Prothrombin When prothrombin is removed from plasma by

adsorption with $Al(OH)_3$, $Ca_3(PO_4)_2$, $Mg(OH)_2$, etc. the treated plasma will not clot on the addition of tissue extract and calcium If prothrombin eluted from the precipitates is added to the absorbed plasma clotting will occur Thus, as would be expected, prothrombin is necessary for the accelerating effect of tissue extracts

Factor V Factor V may be deficient in a blood sample as a congenital abnormality or may be grossly reduced in normal oxalated plasma samples if these are stored for 24 hours or more (see Chapters V and XIV) Such Factor V deficient plasma samples have prolonged clotting times on the addition of tissue extracts and calcium If Factor V is added to deficient blood, a normal clotting time on the addition of tissue extracts and calcium is restored Factor V is therefore also necessary for the normal action of tissue extracts

Factor VII Factor VII may be deficient in the blood as a congenital abnormality or it may be reduced by vitamin K deficiency or treatment with anticoagulants of the dicoumarin type Factor VII differs from Factor V in many respects (see Chapters V and XIV) Factor VII deficient plasma has a long clotting time on the addition of tissue extracts and calcium and if Factor VII is added to the deficient plasma a normal clotting time is restored. Factor VII is necessary for the action of tissue extracts

The Reaction between tissue extracts and Factors V and VII Factors V and VII are not precursors of thrombin but accelerators which affect the speed at which thrombin is formed in the presence of tissue extracts They might act by rendering the prothrombin more active or it might be assumed that the tissue extract is not, by itself capable of converting prothrombin to thrombin but that it requires to react with Factors V and VII before an active substance is found

A very simple experiment suggests that the brain extract reacts with Factors V and VII (Biggs Douglas and Macfarlane 1953b) In this experiment (Table 10) it is shown that the incubation of brain extract with Factors V and VII increases the activity When brain extract and $CaCl_2$ are added to prothrombin and fibrinogen long clotting times are obtained When the brain extract is incubated with the Factors V VII and $CaCl_2$ progressively shortened clotting times are found. These results which have been confirmed and extended by *Hardisty* (1955) suggest that an active prothrombin converting substance is formed by a reaction between Factors V and VII and tissue extracts

Flynn and Coon (1953) and Owren Rapaport, Hjort and Aas

TABLE 10

An experiment to demonstrate a reaction between brain extract and Factors V and VII

In different experiments brain extract was incubated at 37° C with the substances indicated in column 1. At intervals of 1 min 0·1 ml was removed from the incubation mixture and added together with 0·1 ml of M/40-$CaCl_2$ to the substrate indicated for each experiment in column 2. The clotting times of the substrate were recorded in seconds.

The brain extract used was prepared by suspending 0·5 g of dried human brain in 10 ml of 0·85% NaCl.

The factor V was used at a concentration of $\frac{1}{8}$ of that in whole plasma.

The normal serum was used at a dilution of $\frac{1}{5}$ in 0·85% NaCl.

The prothrombin was used at a concentration of about 500 units/ml (50 units in each substrate test). One unit of prothrombin is the amount converted to 1 unit of thrombin, the thrombin unit being approximately the same as the National Institute of Health unit.

The fibrinogen was used at a concentration of approximately 0·6% (a concentration of 0·1% in the substrate mixture).

		Incubation time in minutes					
Incubation mixture	*Substrate*	1	2	3	4	5	6
Brain 0·2 ml 0·85% NaCl 0·4 ml $CaCl_2$-M/40 0·2 ml	Prothrombin, 0·1 ml Fibrinogen 0·1 ml 0·85% NaCl 0·2 ml	85	85	84	88	103	73
Brain 0·2 ml 0·85% NaCl 0·4 ml $CaCl_2$ M/40 0·2 ml	Prothrombin 0·1 ml Fibrinogen 0·1 ml Factor V 0·1 ml Serum 0·1 ml	37	36	33	35	32	27
Brain 0·2 ml Factor V 0·2 ml Serum 0·2 ml $CaCl_2$-M/40 0·2 ml	Prothrombin, 0·1 ml Fibrinogen 0·1 ml 0·85% NaCl 0·2 ml	40	25	20	18	16	15
Brain 0·2 ml Factor V 0·2 ml 0·85% NaCl 0·2 ml $CaCl_2$-M/40 0·2 ml	Prothrombin 0·1 ml Fibrinogen 0·1 ml Serum 0·1 ml 0·85% NaCl, 0·1 ml	55	46	40	49	41	36
Brain 0·2 ml Serum 0·2 ml 0·85% NaCl, 0·2 ml $CaCl_2$ M/40 0·2 ml	Prothrombin 0·1 ml Fibrinogen 0·1 ml Factor V 0·1 ml 0·85% NaCl, 0·1 ml	40	35	32	33	32	27

(1954) have made more detailed studies of these reactions Flynn and Coon showed that after reacting with Factor V or Factor VII

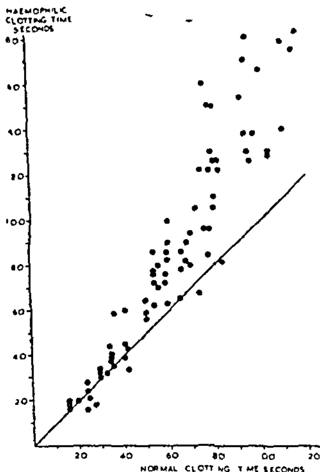


Fig 8 The one-stage test was carried out on many samples of normal and haemophilic plasma using various dilutions of brain thromboplastin. Each point represents the clotting time obtained with a haemophilic sample plotted against the clotting time of a normal sample using the same preparation of thromboplastin in both tests

intermediate products of higher activity can be separated by centrifuging Owren et al have confirmed this finding They believe that a reaction occurs between Factor VII and brain to form a first intermediate product which can be obtained by centrifuging They think

that the increased activity of mixtures of brain and Factor V is due to conversion of Factor V to a more reactive form (proaccelerin is converted to accelerin) and the active substance is adsorbed by brain If the first product between Factor VII and brain (convertin) is mixed with the activated Factor V (accelerin) a final prothrombin converting substance (prothrombinase) which can also be obtained by centrifuging is formed The prothrombinase will clot in the same time as normal plasmas from patients with haemophilia Christmas disease Factor V deficiency and Factor VII deficiency Thus there is good evidence that the tissue extract factor reacts with at least two other blood components (Factors V and VII) before a final prothrombin converting substance is formed

Heparin The addition of heparin to blood lengthens the clotting time on the addition of tissue extracts This effect of heparin is probably due to its action in inhibiting the reaction between thrombin and fibrinogen

Anti Thromboplastin A substance which is thought to inhibit the action of tissue extracts has been extracted from normal blood by Tocantins and Carroll (1949) There is some doubt both as to the *importance of this substance in normal clotting and as to its mode of* action

Antihaemophilic globulin Antihaemophilic globulin is the substance lacking from the blood of haemophilic patients Plasma from these patients has a normal clotting time with concentrated tissue extracts This finding suggests that these extracts supply antihaemophilic globulin or something which will replace it If the extracts are diluted to give clotting times with normal recalcified plasma of more than 40 sec these diluted extracts will not clot haemophilic plasma as well as normal (see Fig 8 and Biggs and Macfarlane 1951) The significance of this finding is not clear

RUSSELL S VIPER VENOM

Russell s viper venom is a very powerful plasma coagulant in the presence of $CaCl_2$ It requires a lipoid for its activity commercial preparations of *lecithin* and *cephalin tissue extracts and platelets* will all serve as the lipoid factor The exact nature of the lipoid activator remains in doubt Poole et al (1955) have shown that the activity of *lecithin is due to a contaminant purified lecithin solutions* are inactive The active lipoid which potentiates Russell s viper

venom is often associated with phosphatidyl ethanolamine (Poole 1955) When a mixture of Russell's viper venom and crude lecithin is added together with $CaCl_2$ to normal plasma clotting times as short as 5 seconds may be recorded. This mixture is more powerful than other extracts which seldom reduce the clotting time to less than 12 seconds If a mixture of Russell s viper venom and crude lecithin is added to normal plasma and to a sample deficient in Factor VII the two samples have the same clotting time on recalcification suggesting that Russell s viper venom differs from brain in that it does not require Factor VII for the acceleration of prothrombin conversion (Rapaport and Owren 1954 Jenkins 1954b)

NOMENCLATURE

According to the classical theory of blood coagulation, prothrombin is converted to thrombin by an enzyme derived from tissues called thrombokinase or thromboplastin. This concept defines thromboplastin as the direct activator of prothrombin But the term is not now used in this sense a substance is said to have thromboplastic activity if it accelerates the clotting of recalcified normal plasma Thus many substances including Russell s viper venom tissue extracts phospholipids platelet suspensions mixture of platelets and serum and mixtures of variously purified clotting factors are all said to provide or produce thromboplastic activity A difficult problem in nomenclature arises either the term thromboplastin can be reserved for the direct activator of prothrombin and all other clot promoting substances including tissue extracts be referred to descriptively or the substances which accelerates the recalcification time of normal plasma can all be said to have thromboplastic activity and a new term or terms can be coined for a specific direct activator of prothrombin.

At present there is much to be said for the second alternative The less academic workers in the field will continue to use the term thromboplastin to denote tissue extracts whatever conclusions may be reached in less practical spheres Many communications about thromboplastin have used the term in the unspecified sense and the experiments have often been so complicated that to disentangle the exact significance of the word thromboplastin as used by all the workers would now be a thankless and unprofitable task. Moreover very little work has been done on the direct activator of prothrombin

and it is therefore relatively easy to define this substance Flynn and Coon (1953) and Owren et al (1954) have isolated a product of reaction between brain extract and Factors V and VII which appears to be a direct activator of prothrombin Owren et al (1954) have used the term prothrombinase for this substance The term is perhaps a little unfortunate in implying that the direct activator is enzymatic when this is not proved but it distinguishes clearly from other factors and we propose to adopt this term

In summary we propose to use the term thromboplastin and thromboplastic activity as a general non-specific designation for all substances which accelerate the recalcification time of normal plasma Prothrombinase is a thromboplastin by this definition but is distinguished from others by being a direct activator of prothrombin it is a final end product while other thromboplastic substances are probably ill defined intermediates or substances which substitute for intermediate forms in the production of prothrombinase For reasons which we hope will be clear later we are restricting the term prothrombinase to the activator derived from tissue extracts and do not at present use it for the activity which develops in blood systems

IS THE TISSUE FACTOR QUANTITATIVELY CONSUMED DURING COAGULATION?

Many workers from the time of Schmidt onwards have thought that tissue extracts are enzymatic in their action Tissue extracts often work at a very high dilution e g brain extracts often have some thromboplastic activity when diluted 10^{-6} From the fact that the active material in tissue extracts can be separated by centrifuging and may therefore have a large molecule it seems unlikely that the highly diluted extract can react stoichiometrically

On the other hand Mertz Seegers and Smith (1939) published an experiment which seemed to show that the amount of thrombin formed from a constant high concentration of prothrombin was proportional to the amount of tissue extract added This experiment was strongly in favour of the view that the tissue factor combined quantitatively with prothrombin At the time that this experiment was made the existence of Factor V was recognized only by Nolf and there is a possibility that Mertz Seegers and Smith s findings are complicated by this factor From its method of preparation the prothrombin that they used was probably almost free from Factor V

Owren has suggested that small amounts of Factor V may have been present in the tissue preparation Factor V is known to influence the yield of thrombin from prothrombin The addition of increasing quantities of extract would, according to this hypothesis involve the addition of increasing quantities of Factor V which would lead to a proportional increase in the amount of thrombin liberated

Experiments since carried out by Seegers and Ware (1948) have not supported Mertz Seegers and Smith s original findings Seegers and Ware have shown that alterations in many factors influence the yield of thrombin from purified prothrombin alterations in the concentration of Factor V and calcium for example have this effect It could be argued that these substances are quantitatively involved in the coagulation process But changes in physical conditions may have a similar effect and it is difficult to see how these can react quantitatively Taking all of these experiments together it appears to Seegers and Ware (1948) that the formation of thrombin from prothrombin may be an equilibrium reaction which can be influenced in one direction or another by many factors This conception is new in that it implies the possibility of the formation of prothrombin from thrombin and such a change has not been demonstrated. The fact that this change has not been observed does not mean that it cannot occur it might be very difficult to detect

Seegers and Ware (1948) have further evidence against the hypothesis that the tissue factor is used quantitatively for thrombin formation. When thromboplastin and calcium are added to a large excess of prothrombin and Factor V thrombin is formed. If the product is centrifuged for four hours at 25 000 r p m. a small sediment is deposited. This sediment can be washed to free it from thrombin and centrifuged again The substance recovered retained much of its activity even when it had been used several times it appears unlikely that the tissue factor could be recovered in this way if it were used up in the course of the reaction

Owren (1947) has also contributed to this subject. Using purified solutions of prothrombin and Factor V he found that if Factor V is in excess varying the concentration of tissue extract does not change the yield of thrombin it alters the speed of thrombin formation. If Factor V is present in limiting amounts then lowering the concentration of thromboplastin decreases the amount of thrombin formed. These experiments can readily be co-ordinated with Seegers and Ware s findings In the original experiments of Mertz, Seegers and

Smith thrombin was formed in the presence of limiting amounts of Factor V, and altering the tissue extract concentration influenced the amount of thrombin formed

In these experiments no account was taken of the fact that tissue extracts must react with at least two factors before prothrombin is converted to thrombin This problem cannot be solved until experiments on prothrombin conversion with prothrombinase have been carried out

THE MEASUREMENT OF THE THROMBOPLASTIC ACTIVITY OF TISSUE EXTRACTS

The activity of different tissue extracts can be compared using the one-stage prothrombin time Various dilutions of the extract are made and each is tested in its ability to shorten the clotting time of *recalcified plasma When this is done it has been found by* Fischer (1935) Feissly (1947) that the clotting time of the plasma bears a definite relation to the concentration of added extract This relation can be expressed by the equation

$$\frac{I}{T} = KC^a$$

where T = the clotting time

C = concentration of extract

K and a are constants depending on the preparation used From relationship it follows that

$$\log T = a \log C\text{-}\log K$$

Thus if the logarithm of the concentration of extract is plotted against the logarithm of the clotting time a straight line graph is obtained With brain extract this relationship holds very well (Fig 9) On this scale if a 1/10 000 dilution of brain thromboplastin is said to contain 1 unit of activity then preparations which give clotting times with normal plasma of 12 14 17 and 26 seconds would have 80 000 45 000 20 000 and 1600 units of activity respectively A value can be attached to normal plasma using the logarithm of the clotting time of the plasma to which saline has been added in place of the extract As a mean of 10 samples the value for normal plasma was 2 units and different samples varied from 0 5-10 units Fig 9 also shows the reaction of haemophilic plasma to dilutions of brain extract The linear relationship holds with haemophilic plasma but

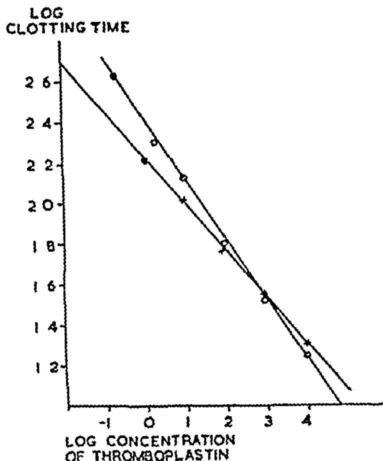


Fig. 9 The one-stage test was carried out on normal (x—x) and haemophilic plasma (O—O) using various dilutions of brain thromboplastin. The logarithm of the clotting time is plotted against the logarithm of the concentration of brain thromboplastin. The clotting times of the normal and haemophilic plasma are indicated on the exterpolations of the lines ●

the slope of the line differs from that of normal plasma. The significance of this difference is not known.

This method of measuring activity has limitations. If the activity of the brain preparation approaches that of normal plasma it causes little change in the clotting time. Thus activity can be measured only if the concentration is great in relation to that of normal plasma. Some brain preparations appear to become inhibitory at very high

concentrations and for assay must be diluted sufficiently to eliminate this inhibitory effect In addition to these limitations it is probable that the method of comparison cannot be applied to preparations from different sources The action of Russell's viper venom on normal plasma, for example, cannot be measured on the scale used for brain extract

The limitations of this method for measuring tissue factor activity are of little importance The method is not used to compare entirely different thromboplastic substances the very weak suspensions are relatively unimportant and any inhibitory effect of strong preparations is obvious

SUMMARY TO CHAPTER IV

Extracts of many tissues accelerate the conversion of prothrombin to thrombin in plasma in the presence of $CaCl_2$ Extracts of different tissues vary quantitatively and qualitatively in the exact mode of action

Experiments with brain extracts have shown that the extract does not convert prothrombin to thrombin directly a reaction occurs between the extract and Factors V and VII and $CaCl_2$ to form a product prothrombinase which is the direct activator of prothrombin

Russell s viper venom differs from the other tissue extracts it requires lipoid for its activity and does not require Factor VII

It is proposed that the term thromboplastin should be used for any substance which accelerates the clotting of recalcified normal plasma Many substances will therefore be said to have thromboplastic activity and the potency of a given preparation can be assessed by its ability to shorten the calcium clotting time of normal plasma The term prothrombinase is used for a substance formed by the reaction of tissue extracts with other factors and is defined as a direct activator of prothrombin

PLASMA AND SERUM ACCELERATORS OF PROTHROMBIN CONVERSION IN THE PRESENCE OF TISSUE EXTRACTS

Although prothrombin is the one essential precursor of thrombin in whole blood various accelerating substances which determine the speed of thrombin formation are necessary for effective coagulation In whole blood thrombin is destroyed as it is formed and the speed of thrombin formation will affect the concentration of thrombin available for the coagulation of fibrinogen (see Chapter XI) The discovery of the accelerators of blood coagulation is important but unfortunately the literature on the subject is confusing and by now at least twenty accelerator substances in plasma or serum have been described The confusion in this subject has arisen almost entirely from the interpretation attached to experimental results Many of the substances described are hypothetical their existence was proposed to explain a variety of otherwise inexplicable phenomena in blood coagulation Much trouble would have been avoided if the various phenomena had been clearly defined and the results of various authors correlated before names had been proposed

At present the best that can be done is to concentrate attention on the actual observations make some attempt to correlate the findings of different authors and emphasize those which may be of immediate practical importance in clinical medicine

ACCELERATORS OF BLOOD COAGULATION FOUND IN PLASMA

In 1908 1928 and 1945 Nolf claimed that prothrombin (which he called thrombozyme) isolated from plasma by adsorption on to $Ca_3(PO_4)_2$ would not form thrombin on the addition of tissue extracts alone but that a substance from the adsorbed plasma (which he called thrombogen) was necessary for thrombin formation According to Nolf's view of blood coagulation thrombogen thrombozyme and fibrinogen combined together to form fibrin and

thrombin appeared as a by-product of the reaction Nolf did not attempt to isolate thrombogen from plasma though his experiments are strongly in favour of the existence of the substance

In 1943 Quick described a phenomenon He observed that if oxalated plasma was stored in the refrigerator the one-stage prothrombin time lengthened progressively and the addition of small amounts of fresh plasma to the stored plasma would correct the abnormality He argued that the deterioration on storage could not be due to destruction of prothrombin because the amount of fresh plasma required to correct the defect contained much less prothrombin than would have been necessary had the defect been due to prothrombin deficiency This argument led Quick to suggest that fresh plasma contains in addition to prothrombin a labile factor which is destroyed on storage Quick's work has been complicated by the discovery that citrated plasma shows little deterioration on storage the labile factor is labile only in the presence of oxalate (Fahey Ware and Seegers 1948) Quick and Stefanini (1948a) have proposed a method for assaying the labile factor in which dilutions of known normal and unknown plasma are compared in their ability to correct the abnormality in stored plasma Using this method Stefanini and Crosby (1950) have claimed that the labile factor is consumed in normal clotting In fact this means that normal serum will not correct the defect in stored plasma to the same extent as normal fresh plasma

Munro and Munro (1947) found that raising the pH of plasma to 10 5 altered its coagulability on the addition of brain emulsion and calcium The defect caused by this treatment was corrected by the addition of serum dicoumarin plasma or plasma adsorbed with $Al(OH)_3$

In addition to these observations three independent groups of workers demonstrated the presence in normal plasma of an accelerator of blood coagulation which was variously called Factor V (Owren 1947) and later renamed pro-accelerin (Owren 1950) ac-globulin (Ware and Seegers 1948a) prothrombin accelerator (Fantl and Nance 1946 and 1948) All of these workers isolated the substance from normal plasma and tested its properties in isolation From Table 11 it is clear that they used similar methods to prepare the accelerator and they used the same methods of demonstrating its activity There is little doubt that they described the same substance It is proposed to adopt Owren's original name Factor

TABLE 11

METHODS USED IN THE PREPARATION OF PLASMA FACTOR V

Author	Source of Blood	Name given to Product	Preliminary Treatment of Plasma			Isolation of activating substance
			Method used to prepare a globulin fraction	Method used to remove fibrinogen	Method used to remove prothrombin	
Owren (1947)	Ox plasma	Factor V	None	Precipitated by 33 saturation with $(NH_4)_2SO_4$	Seitz filtration	Precipitated by 50 saturation with $(NH_4)_2SO_4$ and reprecipitated at pH 5.4
Ware and Seegers (1948a)	Ox plasma or serum	Ac-globulin	Plasma diluted 15 × and globulins precipitated at pH 5.1 Prothrombin together with activators adsorbed on to $Mg(OH)_2$ and eluted with CO_2	Precipitated by 30% saturation with $(NH_4)_2SO_4$	Fractional precipitation with $(NH_4)_2SO_4$	Reprecipitated at pH 5.4
Fantl and Nance (1948)	Human plasma	Prothrombin accelerator	None	None or precipitation by 30% saturation with $(NH_4)_2SO_4$	Adsorption with $Al(OH)_3$ or $BaSO_4$	Precipitation by 45% saturation with $(NH_4)_2SO_4$

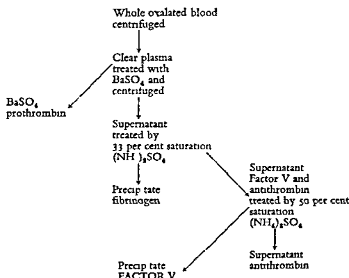
V for this substance because he made the most complete study of its properties and reactions

Factor V, unlike Nolf's thrombogen and Quick s labile factor has been isolated from plasma and studied in isolution by a number of independent workers all of whom have reached the same conclusions This substance is in all probability an essential factor in normal coagulation and its properties and reactions with tissue extracts will be considered in detail

Preparation of Factor V

The details of a simple method for the preparation of Factor V are given in the appendix In principle the method is shown in Table 12 All formed elements are removed from the plasma by centrifuging and prothrombin is adsorbed with $BaSO_4$ The fibrinogen is removed by precipitation at 33 per cent saturation with $(NH_4)_2SO_4$ and the Factor V is obtained by raising the $(NH_4)_2SO_4$ concentration to 50 per cent saturation The final precipitate of Factor V is dissolved in 0 85 per cent saline and dialysed against saline until it is free from $(NH_4)_2SO_4$ The product can be dried by the lyophil drying method but loses much of its activity

TABLE 12



Properties of Factor V

The material prepared by this method from human plasma is usually relatively free from prothrombin, fibrinogen and antithrombin, though the substance prepared from ox plasma usually contains some prothrombin. Naturally the removal of these well recognized components does not mean that the product is 'pure' in that it consists of a single substance. The Factor V (Ac-globulin) prepared by Ware and Seegers (1948a) was tested electrophoretically. The authors say 'The best Ac-globulin preparations we have to date are not stable for sufficient time to maintain full activity during electrophoretic examination.' 'This preparation contained about 5 per cent of prothrombin as impurity.' 'Two major components predominate. One of these move with a mobility of -2.96×10^{-5} which corresponds to beta globulin. The other component gave the mobility -4.73×10^{-5}. It very likely represents Ac-globulin.' If we assume that this rapidly moving component represents Ac-globulin then our product is somewhere in the neighbourhood of 50 per cent pure.

These workers have used the greatest care in the preparation of their accelerator and they are aware that it probably contains 50 per cent of substances other than Ac-globulin (Factor V). There is therefore still much work to be done before Factor V can be said to have been obtained in a pure form.

Factor V deteriorates rapidly on storage and it is destroyed by heating to 50° C for half an hour. The substance is most stable over a pH range from 5 to 9.

Factor V and the conversion of prothrombin to thrombin in the presence of tissue extracts

In a mixture of Factor V, prothrombin and calcium chloride thrombin is formed very slowly or not at all. Tissue extract is necessary for the formation of thrombin from Factor V and prothrombin. In a mixture of prothrombin, Factor V, tissue extract and calcium chloride thrombin is formed rapidly and a greater total amount of thrombin is formed than when Factor V is absent. These results are illustrated in Fig. 2. It appears that Factor V influences both the amount of thrombin formed and the speed of its formation. As an accelerator of thrombin formation from purified prothrombin Factor V is active in a dilution of about 1/500 of the content of normal plasma. Murphy et al. (1948) have shown that the activity

disappears rapidly on storage Owren (1947) finds that Factor V is reduced in serum after storage for three hours to about 15 per cent of that in plasma Factor V therefore appears to be utilized during clotting

The accelerating effect of Factor V is probably due to its reaction together with Factor VII with tissue extracts to form a prothrombin conversion factor prothrombinase (see Chapter IV) In experiments using 'purified' prothrombin and Factor V the Factor VII is supplied in the prothrombin preparations

Occurrence of Factor V

Factor V is present in all normal plasma samples It is present in normal amounts in haemophilia and in the plasma of patients with Vitamin K deficiency It may be slightly reduced in the plasma of patients receiving dicoumarin but it is unlikely that this reduction makes an important contribution to the coagulation defect In liver disease, Factor V may be reduced (Chapter XIV) Cases of naturally occurring Factor V deficiency are described in Chapter XIV

Measurement of Factor V Activity

The most easily demonstrable effect of Factor V is its ability to increase the speed of thrombin formation from purified prothrombin A rough comparison of the amounts of Factor V in two preparations can be obtained by testing the ability of a series of dilutions of the preparations to increase the speed of thrombin formation from the same purified preparation of prothrombin in the presence of calcium chloride and the same tissue extract preparation The degree of acceleration can be judged by either the one- or the two-stage methods These methods are not well adapted to routine use because the preparation of stable purified prothrombin and Factor V is laborious and cases with a primary deficiency of Factor V are rare

If a case of Factor V deficiency is encountered a method of measuring the extent of the defect can be based on the one-stage prothrombin technique used with plasma Mixtures of normal and the Factor V deficient plasma can be made and the mixtures tested by the one-stage method A curve relating concentration of normal plasma and clotting time can then be drawn and the approximate concentration of Factor V deduced

Factor V and other acceleration phenomena

Since Factor V is a well established coagulation factor the question now arises as to whether any of the other less well investigated phenomena can be attributed to its activity The experiments of Nolf (1908 1928 and 1945) already referred to almost certainly involved Factor V Nolf s thrombogen is probably identical with Factor V The term thrombogen cannot be adopted because this designation has been used by Morawitz (1905) for prothrombin Quick s labile factor (1943) is also probably identical with Factor V The labile factor and Factor V both decline in oxalated stored plasma but not in stored citrated plasma Both are destroyed by heating to 56 C both are utilized during coagulation and a purified preparation of Factor V will correct the coagulation defect in stored plasma

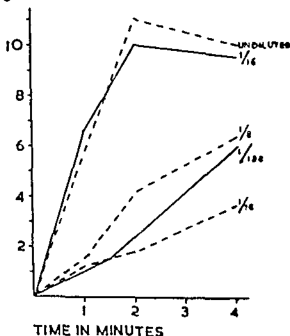


Fig 10. Thrombin formation tested by the two-stage method in mixtures of prothrombin Factor V brain emulsion and calcium chloride The curves drawn in solid lines represent experiments in which the Factor V was supplied by a plasma fraction. The discontinuous lines represent experiments in which Factor V was supplied by platelets

Suspension of platelets and platelet-extracts have an activity resembling that of Factor V when tested with purified prothrombin and brain extract (Ware Fahey and Seegers 1948 Chapter VI) The platelet suspensions washed ten times with saline still retain this accelerator activity If Factor V has been adsorbed from the plasma it is firmly held by the platelets The activity of the platelet suspensions appears to be $\frac{1}{8} - \frac{1}{16}$ of that present in the plasma (Fig 10) Hjort Rapaport and Owren (1954) have shown that platelets from a Factor V deficient patient have no Factor V activity but if they are put into normal plasma they acquire this activity and it cannot be removed by repeated washing It appears that platelets have the ability firmly to adsorb Factor V from plasma

Is Factor V a precursor substance?

Several groups of workers who have studied the activity of Factor V in detail have come to the conclusion that Factor V in plasma is an inactive precursor of an accelerator of blood coagulation which appears for the first time during clotting This opinion is held by Owren (1947 1950) Ware and Seegers (1948b) Alexander and Goldstein (1950) From the following discussion it will be clear that this conclusion rests on indirect evidence

In 1947 Owren made many experiments in which thrombin formation was followed by the two-stage method in mixtures of prothrombin Factor V tissue extract and calcium Owren found that if oxalated plasma was used as the substrate in this test instead of fibrinogen the clotting time of the oxalated plasma was unexpectedly short suggesting that some of the substrate prothrombin had been converted to thrombin Yet the amount of tissue factor and calcium transferred to the substrate was insufficient to convert the prothrombin to thrombin This finding suggests that an active prothrombin converting substance is formed in the incubation mixture originally this was thought to be an active form of Factor V, but it is more likely that the tissue extract had been converted to active prothrombinase in the incubation mixture If Factor V activity was estimated in serum Owren found that immediately after coagulation the apparent amount of Factor V was nearly doubled while three hours later it was reduced to 15 per cent of its original value If coagulation was followed in a mixture lacking Factor V these phenomena were absent From this evidence Owren concluded that Factor V (pro-accelerin) is converted during

clotting to an active substance 'accelerin' which accelerates the conversion of prothrombin to thrombin Following coagulation this substance disappears Again the active coagulant formed might have been any active coagulant formed during clotting including prothrombinase

Ware and Seegers (1948b) found that if serum is tested by the two-stage method for Ac-globulin (Factor V) activity the serum is more effective in proportion to its concentration than plasma in the conversion of prothrombin to thrombin Ware and Seegers believe that this serum accelerator is derived from the plasma Factor V by the action of thrombin They observed that, if just sufficient thrombin was added to ox plasma to coagulate the fibrinogen the amount of Ac-globulin to be detected increased If an excess of thrombin was used, then the amount of Ac-globulin decreased. They conclude that Ac-globulin in plasma is an inactive substance which is converted to an active form in serum Following normal coagulation in which a relatively large amount of thrombin is formed the amount of Ac-globulin to be detected decreases quite markedly In 1950 Alexander and Goldstein confirmed the findings of Ware and Seegers

These observations show that active coagulant substances appear during clotting that thrombin accelerates thrombin formation that Factor V disappears after clotting is complete and that the accelerating effect may not occur if Factor V is absent These results do not prove that Factor V is a precursor substance transformed during clotting into a coagulant though they are consistent with this interpretation

ACCELERATING SUBSTANCES PRESENT IN SERUM

In 1912 Bordet and Delange collected bird and rabbit blood relatively free from tissue contamination and platelets This blood coagulated slowly and yielded a serum which contained a large amount of prothrombin On the addition of platelet extracts thrombin was formed in the serum more rapidly than in the corresponding plasma Bordet and Delange drew the conclusion that the prothrombin in serum was more reactive than that in plasma (proserozyme was converted to serozyme) It is clear that Bordet and Delange s observations have more than one interpretation The

plasma might contain the precursor of an accelerator of blood coagulation which is formed during clotting

Quick and Stefanini (1949a) and Quick and Hussey (1955) have revived Bordet and Delange s view of an inactive precursor of prothrombin as a result of new experiments Quick and Stefanini found that if normal plasma was stored in glass containers the prothrombin isolated from this plasma together with tissue extract and calcium gave a shorter clotting time in the presence of rabbit plasma adsorbed with $Al(OH)_3$ than did the equivalent preparation of prothrombin made from fresh plasma or plasma stored in silicone coated containers Quick and Stefanini claim that this observation indicates a change in reactivity of prothrombin, in other words the prothrombin in fresh plasma differs from that in plasma stored in glass The fresh plasma contains an inactive precursor of prothrombin which is converted to the active form by glass contact This hypothesis was supported by the discovery of a patient with an abnormal one-stage prothrombin time who was found to have a normal amount of prothrombin by Quick s test when the plasma was stored in glass An abnormal proportion of prothrombin was in the precursor form Quick s hypothesis is ingenious and it may be the true explanation of his findings but it must be emphasized that his findings could have other explanations The prothrombin isolated by adsorption may include an accelerator of blood coagulation which is activated on storage in glass containers In fact this conclusion is favoured by Alexander and Landwehr (1949a) who carried out experiments on stored plasma which confirm Quick s findings Alternatively storage in glass may remove a coagulation inhibitor as suggested by Tocantins (1945 etc)

In 1948 Owen and Bollman and in 1951 Owen Magath and Bollman found that prothrombin free serum would accelerate thrombin formation from the plasma of patients under treatment with dicoumarin This finding which was confirmed by Biggs (1951) suggested that dicoumarin plasma lacks a prothrombin conversion factor which is present in normal serum This factor cannot be Factor V because Factor V is not diminished in dicoumarin plasma and is absent from serum

de Vries et al (1949) and Alexander et al (1949) found that if plasma was diluted 1 20 with $BaSO_4$ adsorbed plasma the clotting time of the mixture in the presence of tissue extract and calcium chloride was shortened considerably by the addition of 1/20 volume

of normal serum The mixture of normal plasma and $BaSO_4$ adsorbed plasma contains an excess of Factor V thus the shortening effect of serum could not be due to Factor V activity These workers found that the amount of acceleration produced by serum was inversely related to the amount of prothrombin contained in the serum If much prothrombin remained in the serum then there was little accelerating activity It is inferred from these results that an accelerator of coagulation appears during the coagulation process This accelerating effect is removed from serum by adsorbtion with $BaSO_4$ The accelerating effect of the serum of patients receiving dicoumarin is less than that of normal serum

Owren (1950a 1951 1952) has postulated a substance in serum called proconvertin This substance is proposed from several lines of research When ox plasma is passed through a Seitz filter the treated plasma will not clot on the addition of tissue extract and calcium Since the filtered plasma contains an excess of Factor V it was usually assumed that the failure of thrombin formation is due to the absence of prothrombin But Owren found that in certain experiments a mixture of filtered plasma serum tissue extract and calcium clotted rapidly This rapid clotting indicated that either the filtered plasma or the serum must have contained prothrombin Initially Owren (1950a) favoured the view that normal serum contained prothrombin and that the filtered plasma contained an essential activator of prothrombin Later experiments (Owren 1951 1952) showed that this view was wrong The filtered plasma contains prothrombin which cannot be converted to thrombin in the presence of Factor V brain extract and calcium An essential activator (proconvertin) was removed from the plasma by Seitz filtration but was present in serum Owren (1950a 1951 1952) holds the view that during clotting an active coagulant convertin is formed from proconvertin This active substance disappears quite rapidly from serum leaving an excess of residual proconvertin This convertin phenomenon may of course often be due to the presence of prothrombinase Proconvertin is deficient in the plasma of patients treated with drugs of the dicoumarin group

Owren (1950a) has demonstrated the conversion of proconvertin to convertin in the plasma of a patient deficient in Factor V If calcium chloride is added to this patient s plasma clotting is delayed but it can be shown that some change is occurring in the plasma because when Factor V is added to the mixture of Factor V deficient

plasma and calcium the clotting time of the mixture shortens progressively as the time of incubation with calcium is increased (Table 13) 'Proconvertin is adsorbed from serum with $Al(OH)_3$ and $BaSO_4$ and thus differs from Factor V which is not adsorbed by these substances According to Owren convertin is formed from a reaction between tissue extract proconvertin and $CaCl_2$ (Owren et al 1954 see also Chapter IV)

TABLE 13

THE EFFECT OF INCUBATING THE PLASMA FROM A PATIENT DEFICIENT IN FACTOR V WITH CALCIUM OR CALCIUM AND TISSUE EXTRACT ON THE CLOTTING TIME ON THE ADDITION OF FACTOR V (FROM OWREN 1950a)

Parahaemophilia plasma +Ca	*Without Factor V*	10 *minutes*
	Incubation time before addition of Factor V	*Clotting time*
Addition of Factor V	0 sec 30 60 90 120	69 sec 39 30 27 25
Parahaemophilia-plasma+ tissue extract Dil 1 50+ Ca	*Without Factor V*	120 *sec*
	Incubation time before addition of Factor V	*Clotting time*
Addition of Factor V	0 sec 15 30 45	28 sec 16 11 4 11 2

Koller et al (1951 1952) have made experiments on a substance which they call 'Factor VII Factor VI being a term already used by Owren for a substance of not very clearly defined indentity which may be the same as prothrombinase This Factor VII is certainly the same as Owren s proconvertin The experiments of Koller et al show quite conclusively that Factor VII is an accelerator of prothrombin conversion in the presence of tissue extracts

These lines of experiment were considered because they have features in common The serum accelerators described by Owen

and Bollman (1948) Alexander and co-workers (1949a) Owren (1950a and b 1951) and Koller et al (1951 1952) are all adsorbed by $BaSO_4$ and have a demonstrable effect in the presence of an excess of Factor V and are deficient in the plasma of patients treated with dicoumarin In patients treated with dicoumarin there is often a dissociation between the reduction of prothrombin and the accelerator indicating two independent deficiencies In the early stages of treatment there may be a greater reduction of the accelerator than of prothrombin (Owen and Bollman 1948 Owren Magath and Bollman 1951 Koller et al. 1951)

The serum accelerator appears to be a definite substance Its effect has been observed by several groups of workers who are reasonably agreed on the interpretation of its properties The substance is deficient in the blood of patients treated with the dicoumarin group of drugs and, as will be shown later (Chapter XIV) prothrombin may be deficient when the accelerator is little altered in quantity

Nomenclature

The serum accelerator has been called a prothrombin conversion factor (Owen and Bollman 1948) serum prothrombin conversion accelerator or S P C A (Alexander et al 1949a and de Vries et al 1949) proconvertin (Owren 1950a 1951) and Factor VII (Koller et al 1951) It is proposed to use the term Factor VII suggested by Koller et al The names used by Owen and Bollman (1948) and Alexander et al (1949a) have precedence but they are not very satisfactory Owen and Bollman s term prothrombin conversion factor could equally be applied to Factor V The term S P C A used by de Vries et al is sufficiently specific but the experiments of de Vries et al were carried out with the one-stage prothrombin method and the system used for the measurement of S P C A is based on the assumption that this method can give a quantitative measurement of prothrombin Actually this method of measurement precludes the possibility of detecting S P C A activity in normal plasma Owren s conception of proconvertin and convertin seems to us to be premature Convertin activity is probably often due to the presence of a not very clearly defined intermediate product in the formation of the direct activator of prothrombin or to the presence of prothrombinase itself The nature of the reactions by which intermediate

products are formed *during normal clotting* and the relation of these products to coagulants derived from tissue extracts is in our opinion still obscure Koller et al (1951) gave the first definite evidence that a serum factor accelerates prothrombin conversion in the presence of tissue extracts Their term Factor VII is sufficiently specific for present purposes without implying the occurrence of any specified reaction

Mode of Action of Factor VII

Koller et al (1951) and Owren (1950a and 1951) have shown that in the absence of Factor VII prothrombin will not react with Factor V brain extract and $CaCl_2$ Koller et al (1951) have shown that the concentration of Factor VII has a marked effect on the speed of prothrombin conversion but had no effect on the amount of thrombin formed

Analysing the nature of the accelerating effect of Factor VII most authors are now agreed that some reaction between the tissue extract and Factor VII occurs Jacox (1941) showed that the coagulant activity of brain extract was increased by incubating the extract with normal serum Mann and Hurn (1951) have also demonstrated a reaction between brain extracts and some plasma or serum factor Owren (1950a 1951 1955) has suggested that Factor VII reacts with brain to form a specific intermediate product convertin Biggs Douglas and Macfarlane (1953b) and Hardisty (1955) Flynn and Coon (1953) Owren et al (1954) have presented evidence to show that both Factor V and Factor VII react with brain extracts to form a final prothrombin converting substance or prothrombinase (see Chapter IV) These experiments are essentially in agreement with the general view put forward by Owren in 1955 The only difference of opinion between ourselves and Owren is that we hesitate at present to be specific about the exact nature of the substances formed as intermediate products in the formation of prothrombinase from tissue extracts nor are we certain that the tissue activator is identical with the activator derived from blood constituents

The Measurement of Factor VII activity

Alexander et al (1949a) and de Vries et al (1949) have measured the effect of the serum accelerator by the extent to which serum shortens the one-stage clotting time of a mixture of normal plasma and plasma treated with $BaSO_4$ Since $BaSO_4$ adsorbs Factor VII and prothrombin the substrate for the test (normal plasma diluted

with $BaSO_4$ treated plasma) is deficient in both of these substances Alexander et al assume that the extent to which increasing concentrations of plasma shorten the one-stage clotting time of mixtures of normal plasma and $BaSO_4$ treated plasma will be a measure of the prothrombin content of the plasma Since serum contains little prothrombin the shortening of the clotting time caused by serum cannot be attributed to prothrombin but must be due to the accelerator Thus the amount of Factor VII is measured as a difference between the reduction expected from the amount of prothrombin in the serum and the actual reduction observed. This method assumes that the one-stage method as carried out by Alexander et al gives a measure of prothrombin an assumption which is not well founded Normal plasma contains Factor VII in addition to prothrombin and since the substrate is deficient in both Factor VII and prothrombin the shortening of the clotting time caused by normal plasma may be due to the addition of either prothrombin or of Factor VII

Factor VII is greatly reduced in the plasma of patients treated with drugs of the dicoumarin group During the first few days of treatment prothrombin is not significantly abnormal During this period of treatment plasma samples with a long one-stage prothrombin time may be used to test the extent of Factor VII deficiency in an unknown specimen Proportional mixtures of normal plasma and the plasma of the patient treated with the anticoagulant are made and tested by the one-stage method. Similar mixtures of the plasma to be tested with the plasma from the anticoagulant treated patient are also tested and the two sets of results compared. Owren 1950 has used a similar method using the plasma of a patient with a congenital deficiency of Factor VII

Koller et al (1951) and Owren and Aas (1951) have devised a method for measuring Factor VII based on the use of Seitz filtered ox plasma. With carefully controlled conditions it is possible to prepare Seitz filtered ox plasma which contains prothrombin but which lacks Factor VII Using the one-stage method the amount of Factor VII present can be recorded by testing the ability of normal and a similar abnormal specimen to shorten the clotting time of the Seitz filtered ox plasma

The Properties of Factor VII

Factor VII is a protein found in the beta-globulin fraction of

normal plasma and serum (Owen and McKenzie 1954) It is adsorbed from normal plasma and serum by inorganic precipitates such as $BaSO_4$ $Al(OH)_3$ $Ca_3(PO_4)_2$ etc It is heat labile and destroyed by pH3 Factor VII activity is difficult to extract from plasma because by most fractionation methods prothrombin and Factor VII are precipitated or adsorbed together Koller (1954) has developed a chromatographic method by which prothrombin and Factor VII can be separated from each other

Factor VII accelerates the conversion of prothrombin to thrombin in mixtures containing Factor V, tissue extracts prothrombin and $CaCl_2$ Factor VII is deficient in the blood of patients treated with the dicoumarin group of drugs in vitamin K deficiency in liver disease in haemorrhagic disease of the newborn and occasionally as a congenital defect (see Chapter XIV)

Is Factor VII present in plasma as a precursor substance?

Since Factor VII and prothrombin are separated only with difficulty in plasma Factor VII activity is often studied in serum The use of substrates deficient in Factor VII but containing adequate amounts of prothrombin facilitates the study of Factor VII in plasma Using the plasma of a patient treated with tromexan (deficient in Factor VII) the Factor VII activity of plasma and serum were compared (Table 14) *It will be seen that the addition of* a small amount of serum causes a far greater reduction of the one-stage prothrombin time than does the addition of the same amount of

TABLE 14

THE EFFECT OF ADDING VARIOUS ACCELERATING SUBSTANCES ON THE ONE-STAGE PROTHROMBIN TIME OF TROMEXAN PLASMA

Source of accelerator	*Dilution of accelerator added*						*No accelerator*
	1/10	1/ o	1/40	1/80	1/160	1/320	
Normal Plasma	20	22	22	23	25	26	26
Prothrombin deficient plasma	0	23	24	25	25	27	26
Normal serum	14	16	19	21	22	23	26
Serum from prothrombin deficient patient	15	16	19	21	2	23	26

plasma It will also be noted in this table that the plasma and serum of a prothrombin deficient patient have the same effect as normal plasma and serum It would appear that the Factor VII activity of serum is greater than that of plasma

If the serum is derived from blood collected into silicone coated tubes the activity of the serum judged by its ability to shorten the one-stage clotting time of Factor VII deficient plasma is the same as that of plasma. The additional activity was developed in the serum of the prothrombin deficient patient only if the blood was shaken with glass beads during clotting Haemophilic serum develops activity superior to that of the plasma. On the few occasions tested Christmas disease serum appears rather inferior to normal serum though the plasma activities are comparable These results suggest that contact with glass during clotting makes some change in Factor VII which appears to be in a more active state in serum

Rapaport et al (1954) have demonstrated clearly that contact with glass affects the Factor VII activity of plasma Contact with glass causing a marked increase in Factor VII activity when comparison is made with the same plasma collected and stored in a silicone coated vessel These results are certainly to be attributed to the same cause as those described by Quick and Hussey (1955) and Alexander and Landwehr (1949a) already referred to Quick and Hussey conclude that the changes in one-stage prothrombin time which occur on contact with glass are due to an alteration in the reactive state of prothrombin The decision between these two views rests on some method other than the one-stage prothrombin time for measuring prothrombin Rapaport et al (1954) have used a quantitative method for prothrombin determination in their experiments their interpretation of the results is therefore the more probable confirming the opinion of Alexander and Landwehr (1949a)

These results suggest that during clotting in contact with glass or on prolonged exposure of plasma to glass some increase in Factor VII activity develops Whether or not this change is due to the activation of Factor VII or the removal of some inhibitory factor has not been determined The results given in Table 13 also demonstrate that the prolonged one-stage prothrombin time caused by the dicoumarin drugs is due mainly to Factor VII deficiency The clotting time is more greatly shortened by serum (which contains no prothrombin) than by plasma and a prothrombin deficient sample behaves in the same manner as the normal

From this general confusion two factors or sets of consistent phenomena emerge Factor V is a labile substance which is consumed during clotting is not adsorbed by $Al(OH)_3$ $BaSO_4$ $Ca_3(PO_4)_2$ etc Factor V is required for the rapid conversion of prothrombin to thrombin with brain extract and $CaCl_2$ Factor VII is a stable substance which may be present in plasma in an inactive form it is present in serum and therefore not consumed during clotting it is adsorbed by $Al(OH)_3$ $BaSO_4$ $Ca_3(PO_4)_2$ and is a beta-globulin This substance is also required for the rapid conversion of prothrombin to thrombin by brain extract and $CaCl_2$ Natural deficiencies of both Factor V and VII are described (see Chapter XIV)

SUMMARY TO CHAPTER V

Two Factors Factors V and VII appear to be necessary for the rapid conversion of prothrombin to thrombin in the presence of tissue extracts and $CaCl_2$

Factor V is obtained from plasma it is not readily adsorbed by $Al(OH)_3$ $BaSO_4$ etc It deteriorates in stored oxalated plasma and is precipitated by 50 per cent saturation with $(NH_4)_2SO_4$ Factor V is used up during clotting and is therefore absent from normal serum Factor V is probably synonymous with Nolf s Thrombogen with the labile factor with plasma accelerator globulin and with pro-accelerin

Factor VII is usually prepared from normal serum where it is to be found in the beta-globulin fraction It is strongly adsorbed by $Al(OH)_3$ $BaSO_4$ etc It is not used up during clotting Factor VII is deficient in the plasma of patients treated with the dicoumarin anticoagulants Factor VII is probably synonymous with the stable factor serum prothrombin conversion accelerator (S P C A) cothromboplastin and proconvertin

Factors V and VII probably react with tissue extract in the presence of $CaCl_2$ to form a prothrombin converting substance prothrombinase

CHAPTER VI

PLASMA THROMBOPLASTIN

In the classical scheme of blood coagulation the factor which begins the whole process of clotting is thromboplastin and it is often tacitly assumed that this is derived from damaged tissues But normal blood taken with care to avoid the least contamination with tissue fluid, clots firmly and rapidly It is clear that it must contain an intrinsic source of thromboplastin which is activated following contact with a foreign surface Thus intrinsic thromboplastin would seem to be of greater physiological importance than the tissue extracts which have been studied so extensively since the latter probably have little chance of participating in natural clotting The intrinsic thromboplastin is contained in the plasma, since plasma clots as readily as blood on contact with glass and throughout this chapter therefore, it is referred to as 'plasma thromboplastin The general non-specific term thromboplastin is used rather than prothrombinase (see Chapter IV) because it is not yet certain whether or not the plasma prothrombin activator is a single substance or a mixture and whether or not the final prothrombin activator is identical with that formed from tissue extracts

The origin of plasma thromboplastin is difficult to study because the development of thromboplastic activity in plasma is far removed from the thrombin-fibrinogen reaction the indicator by which all reactions of coagulation must be measured The idea that the plasma itself might contain in an inactive form its own thromboplastin which was activated as the first stage in the process of coagulation was put forward quite clearly by Collingwood and MacMahon (1912) They considered that a precursor of thromboplastin which they called prothrombokinase was derived from the platelets or from small platelet-like bodies in the plasma Thus prothrombokinase was activated in some way as the initial stage in coagulation and it is interesting that they observed that this process was hastened by the addition of serum which they supposed provided either pre-formed thromboplastin or some activator of prothrombokinase Curiously the idea of potential thromboplastic activity residing in the plasma received very little serious attention until comparatively

recently though certain authorities including Nolf and Lenggenhager have always maintained that the plasma contains all that is necessary to initiate its own coagulation on contact with a foreign surface The nature of a possible mechanism for generating thromboplastin was seldom considered

A study of this problem can be made by considering the clotting time of citrated plasma to which calcium chloride has been added In experiments with prothrombin and Factor V separated from plasma no thrombin is formed unless some thromboplastin is present Since citrated plasma clots on the addition of calcium it must be presumed that some thromboplastin is present The addition of tissue extracts to normal plasma greatly decreases the clotting time in the presence of calcium chloride and it may reasonably be thought that thromboplastin is a limiting factor in the clotting of normal plasma If thromboplastin is the main limiting factor a value for the thromboplastin content of plasma may be derived from the logarithm of its clotting time as suggested in Chapter IV According to this criterion the thromboplastin content of normal plasma would be equivalent to a 1/10 000 dilution of a brain suspension which undiluted clots normal plasma in twenty seconds

THE PLATELETS AND PLASMA THROMBOPLASTIN

Bordet and Delange (1912) thought that the plasma thromboplastin came from platelets They found that the plasma of birds freed from the cells comparable to platelets became incoagulable The plasma of rabbits collected into paraffin-coated containers and freed by centrifuging from most of the platelets clotted slowly and in this plasma little of the prothrombin was converted to thrombin when coagulation was complete A large amount of prothrombin could be found in the serum derived from the clotting of platelet poor plasma twenty-four hours after coagulation

If any analogy can be drawn from bird blood this evidence suggests that platelets are a source of thromboplastin But if platelets constitute the thromboplastin of plasma they behave quite differently from brain extracts because an increase in the platelet number above that occurring in normal plasma does not lead to a corresponding decrease in clotting time Increase in platelets does not decrease the clotting time of normal plasma to less than about sixty to eighty seconds whereas with brain extract it is possible to

obtain clotting times of twelve seconds Moreover if whole plasma containing its normal number of platelets is collected into a silicone container coagulation is slow, and little prothrombin is converted to thrombin although the normal number of platelets is present

These experiments suggest a number of possible conclusions If platelets are the source of thromboplastin in normal plasma then thromboplastin is not the limiting factor in coagulation because increase in platelet number does not lead to an equivalent shortening of the clotting time Alternatively the thromboplastin in plasma has more than one component Platelets may be one component which at or below their normal level is a limiting factor in normal clotting The second component becomes a limiting factor when platelets are increased Another conclusion which seems to follow from the experimental results is that platelets as they occur in the circulating blood have much less activity than platelets which have been exposed to glass Or alternatively a silicone surface inhibits thromboplastic activity

Another index of plasma thromboplastic activity is the conversion of prothrombin to thrombin during coagulation When normal whole blood clots in a glass tube most of the prothrombin is converted to thrombin by the plasma thromboplastic system When thrombocytopenic blood clots then much prothrombin remains in the serum (Bordet and Delange 1912 Quick 1947a Brinkhous 1947) From this criterion platelets must contribute to thromboplastic activity

Further information about platelet thromboplastin can be obtained from experiments on relatively purified coagulation factors When Factor V prothrombin and calcium chloride are incubated together very little thrombin is formed unless some thromboplastin is added The ability to accelerate thrombin formation from Factor V and prothrombin may be used as an indication of thromboplastic activity Platelets added to Factor V and prothrombin stimulate slow thrombin formation suggesting some thromboplastic activity (Fig 11) These findings agree with those of Ferguson (1949) In terms of brain extract this activity is very slight and is probably comparable to a 1/100 000 dilution of a brain preparation which clots normal plasma in 20 seconds when undiluted This platelet thromboplastin resists boiling In addition it has been found (Ware Fahey and Seegers 1948) that platelets have Factor V activity *in that they*

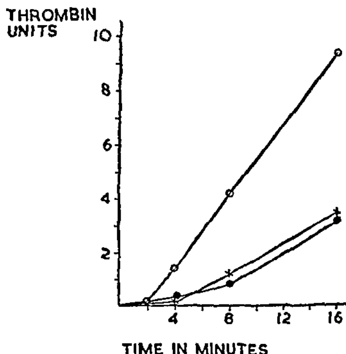


Fig 11 The formation of thrombin was followed by the two-stage method in mixtures of prothrombin Factor V platelets and calcium chloride (●—●) The prothrombin Factor V and platelets were prepared from haemophilic plasma. In one experiment (×—×) the fibrinogen fraction from haemophilic plasma was added to the mixture *In a second* experiment (O—O) the fibrinogen fraction of normal plasma was added

will accelerate thrombin formation in a mixture of prothrombin brain and calcium chloride (Chapter V)

Tested in isolation platelets have two distinct accelerating effects on thrombin formation of these two effects the Factor V activity is much the more potent The thromboplastin activity is slight and there has been some doubt as to whether or not this very small activity can play any significant part in normal thrombin formation (*Ware Fahey and Seegers 1948*) *It is certainly difficult to see* how so small an amount of thromboplastin could have much effect in the presence of potent plasma antithrombin and possibly also antithromboplastin

TWO COMPONENTS OF THROMBOPLASTIN

Macfarlane (1942) suggested that two factors were involved in thromboplastin formation a lipoid substance derived from the

platelets and possibly existing in other forms in the plasma and an enzyme-like labile factor On contact with a foreign surface some reaction occurred resulting in the formation of active thromboplastin This supposition was based by analogy on the observation that Russell's viper venom which has a powerful thromboplastin-like action is greatly potentiated by tissue lipoids (Trevan and Macfarlane 1936b) and is incapable of coagulating plasma from which all platelets and lipoid have been removed (Macfarlane Trevan and Attwood 1941) The observation that human saliva contains a labile coagulant similarly dependent for its action upon the presence of lipoid or platelets suggests that a factor acting like Russell's viper venom is a physiological component of human tissues and may well exist in plasma

ANTIHAEMOPHILIC GLOBULIN AND PLASMA THROMBOPLASTIN

Platelet rich haemophilic plasma has a long clotting time on recalcification and, since it is rapidly clotted by tissue extracts it must be concluded that haemophilic plasma is deficient in plasma thromboplastic activity The platelets of haemophilic plasma are apparently normal because they are as efficient as normal platelets in converting the prothrombin of thrombocytopenic blood to thrombin (Quick 1947 Brinkhous 1947) Quick and Brinkhous found that if platelets were removed from haemophilic plasma and from normal plasma by high speed centrifuging in silicone coated apparatus the haemophilic platelet-free plasma would clot normally on the addition of either normal or haemophilic platelets provided that a small proportion of normal plasma was also added It therefore appears that the presence of platelets and of normal plasma containing antihaemophilic factor is essential for the coagulation of haemophilic blood Quick concluded from this experiment that normal plasma contains a precursor of thromboplastin which he termed thromboplastinogen' and that this is activated by a factor derived from platelets which disintegrate on contact with a foreign surface Brinkhous supposed that the platelets contained thromboplastin and that their disruption is brought about by a plasma factor which is activated by contact with a foreign surface and which he called thrombocytolysin' Both workers agreed that it is the plasma factor which is deficient in haemophilia, in other words that the antihaemophilic factor reacts in some way with the platelets after contact with a foreign surface The result of this reaction is the development of thromboplastic

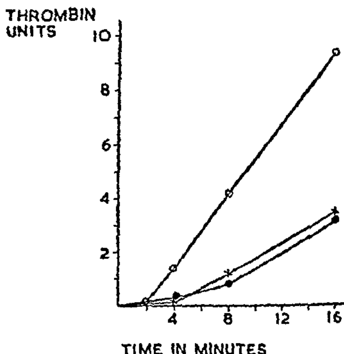


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activity Quick (1951a) has further suggested that platelet disruption is facilitated by thrombin so that the process of coagulation is self-accelerating Shinowara (1951a and b) has carried these investigations a stage further by separating what he considers to be the two components of blood thromboplastin One of these is obtained from the blood cells including the platelets and is a lipoid or lipoprotein the other component is present in the plasma and is a heat labile protein The two factors together form active thromboplastin but are inactive separately and appear to combine stoichiometrically He suggests that the labile plasma component is identical with antihaemophilic globulin and has shown by quantitative separation that it is deficient in haemophilia

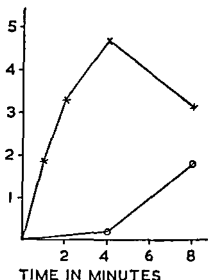


Fig 12 The formation of thrombin was followed by the two-stage method in a mixture of prothrombin Factor V fibrinogen platelets and calcium chloride (O—O) The experiment was then repeated with a final concentration of 0 008 units of thrombin in the incubation mixture (×—×)

Graham Penick and Brinkhous (1951) have shown that antihaemophilic globulin appears to be consumed during the coagulation of normal dog blood so that very little remains in the serum Its consumption appears to run parallel to the consumption of prothrombin and thrombocytopenia or absence of contact with a

foreign surface which reduce prothrombin consumption also reduce antihaemophilic factor consumption Thus may mean that prothrombin consumption is dependent on antihaemophilic factor consumption or that antihaemophilic factor is consumed in some process which is itself dependent on the products of prothrombin conversion Our own observations on the activity of antihaemophilic factor in thrombin generation are described in a later section

Experiments with isolated coagulation factors have shown that the fibrinogen fraction of plasma which contains the antihaemophilic globulin increases the thromboplastic activity of platelets (Fig 11) This experiment also supports the view that the antihaemophilic globulin is important in the formation of plasma thromboplastin

In experiments with relatively purified coagulation factors the presence of a minute trace of thrombin accelerates thrombin formation from prothrombin (probably containing Factor VII) Factor V fibrinogen and platelets (Fig 12) Thus plasma thromboplastin like *tissue thromboplastin is more effective in the presence* of thrombin.

PLASMA THROMBOPLASTIN AND THE GENERATION OF THROMBIN

The activity of the plasma thromboplastin system can be studied further using the two-stage method in plasma or whole blood In Fig 13 the effect of adding a low concentration of brain extract to plasma is shown A 1/1000 dilution of brain extract shortened the clotting time of the plasma from 2-3 minutes to about 1 minute yet the general pattern of thrombin formation tested by the two-stage method is similar to that in the specimen to which no tissue extract had been added. Examining this figure the striking feature of the experiment in which no extract was added is a delay of about 2 minutes when no thrombin can be detected. After this delay there is a rapid generation of thrombin If this rapid thrombin generation can be taken to indicate the appearance of intrinsic thromboplastic activity of plasma then clearly it must be a very powerful thromboplastin to cause such an explosive generation of thrombin When the 1/1000 dilution of brain extract was added to the plasma its main effect was to shorten the delay phase but not noticeably to alter the general pattern of thrombin formation In haemophilic plasma

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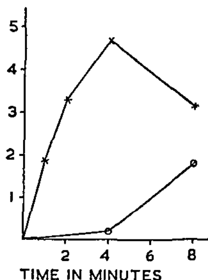


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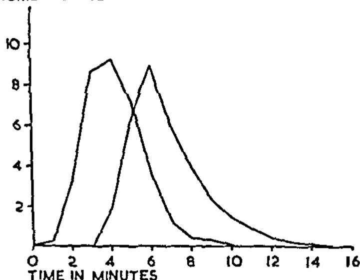


Fig 54. The thrombin generation test was carried out on normal whole blood in glass tubes (Appendix IV 27) with and without the addition of thrombin to the blood. The presence of thrombin greatly shortens the delay phase before rapid thrombin formation occurs.

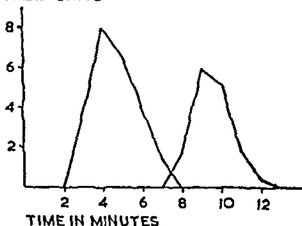


Fig 55 The thrombin generation test was carried out on normal whole blood collected into a silicone coated tube with and without the addition of 10 per cent of normal serum. The presence of serum shortens the delay phase before rapid thrombin formation occurs.

the intrinsic thromboplastic activity of plasma as demonstrated by the two-stage method is absent.

The development of this intrinsic thromboplastic activity can be studied in whole blood or in plasma separated in silicone-coated containers with no added anticoagulant (See Appendix IV thrombin generation test) In whole blood the general pattern of thrombin

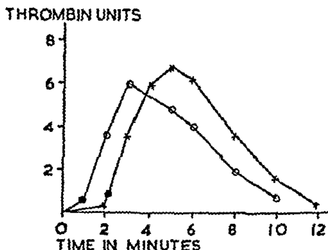


Fig 13 The two-stage test was carried out on two mixtures. (1) Normal plasma saline and calcium chloride in equal parts (× — ×) (2) Normal plasma brain emulsion diluted 1/1000 and calcium chloride in equal parts (O — O) The one-stage clotting times of the mixtures are shown ●

formation is similar to that in recalcified citrated plasma There is a marked delay phase in which no thrombin can be detected which lasts until coagulation occurs After clotting starts there is a rapid thrombin generation (Fig 14) If thrombin is added to the whole blood the delay in thrombin formation is greatly shortened (Fig 14) Normal serum which has no ability to clot fibrinogen will also greatly reduce the delay before rapid thrombin formation (Fig 15)

The progress of thrombin formation can also be followed in whole unaltered plasma obtained from blood collected in silicone-coated containers If the majority of platelets are removed from this plasma there is no explosive thrombin generation and prothrombin remains in the serum for long periods of time after clotting is complete Similarly in the plasma of haemophilic patients in whom Lee and White clotting time exceeds thirty minutes there is no explosive liberation of thrombin

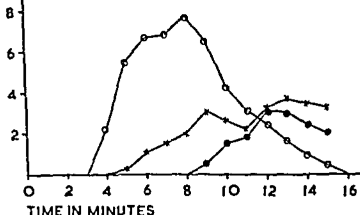


Fig 17 The thrombin generation test was carried out on haemophilic plasma to which varying proportions of normal plasma had been added. ●—● 1 per cent of normal plasma. x—x 10 per cent of normal plasma. O—O 20 per cent of normal plasma.

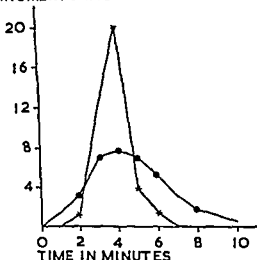


Fig 18 The thrombin generation test was carried out on normal platelet poor plasma to which platelets (x—x) or brain thromboplastin diluted 1/10 (●—●) had been added.

From these experiments it would appear that both platelets and antihaemophilic globulin are necessary for the initiation of the blood thromboplastic activity. Using the technique with whole untreated plasma *it can be shown that the number of platelets influences* the amount of thrombin formed in the explosive phase and the amount of prothrombin remaining after coagulation but not the time at which thrombin formation occurs (Fig 16) The amount of

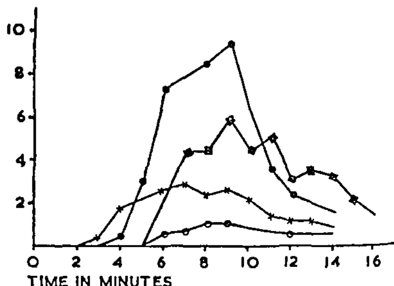


Fig 16 The thrombin generation test was carried out on normal plasma prepared by centrifuging normal blood collected into a silicone coated container The number of platelets in the plasma was varied by mixing plasma samples which had been centrifuged at different speeds The proportion of platelets present directly affects the amount of thrombin formed. O—O platelet poor plasma ●—● platelet rich plasma ×—× 10 per cent of platelet rich plasma ■—■ 20 per cent of platelet rich plasma

antihaemophilic globulin influences the time at which the explosive phase of clotting occurs (Fig 17)

When thrombocytopenic blood clots much prothrombin remains in the serum but some change occurs in the blood because the addition of platelets after coagulation is complete leads to a very rapid thrombin formation The initial delay phase disappears A similar change occurs in haemophilic blood. When the coagulation of haemophilic blood is complete the addition of antihaemophilic material is followed by a rapid thrombin formation with little or no

that the blood thromboplastin is qualitatively similar to brain but much weaker in strength The blood thromboplastin appears to be as effective as strong suspensions of brain extract but the initial delay phase which precedes its effect has led to under-estimation of its strength

THE DIRECT MEASUREMENT OF PLASMA THROMBOPLASTIC ACTIVITY

Any further progress in the study of plasma thromboplastic activity requires a more specific method for its measurement. The method of studying thrombin formation has the disadvantage that thrombin is always present in the system together with thromboplastin and the relative importance of the two coagulants cannot easily be differentiated. Moreover further advances with this technique require a supply of blood from a variety of patients with rare deficiencies such as Factor V and Factor VII deficiency

The study of a patient with a deficiency of prothrombin whose blood apparently contained all other known clotting factors gave the clue to a more general method of recording plasma thromboplastin formation

When calcium and a physiological level of platelets were added to a 1/5 dilution of this patient s plasma a very powerful thromboplastin was formed (Table 15) This thromboplastin was formed gradually an incubation of about four minutes being required for the full activity to appear The thromboplastic activity was tested by adding 0 1 ml amounts of the incubation mixture to 0 1 ml amounts of citrated normal plasma from which the most of the platelets had been removed by centrifuging The mixtures were immediately recalcified with 0 1 ml of calcium chloride The substrate clotting times therefore represent one-stage prothrombin times Using this patient s plasma little thrombin was formed (Table 15) The substrate clotting times could not therefore be attributed to the presence of thrombin The potency of this thromboplastin hardly needs emphasis from platelets at a level of about 100 000 cu. mm. in the incubation mixture and a 1/15 final dilution of plasma a coagulant of strength comparable to that of concentrated brain preparations was formed. This thromboplastic activity is unstable by one hour after clotting much of the activity had dis-

delay phase Other authors have also noted a shorter one-stage prothrombin time in the serum (to which fibrinogen is added) compared with the plasma of patients with haemophilia or thrombocytopenia It would seem that this abolition of the delay phase cannot be directly connected with either platelets or antihaemophilic globulin because it will occur when either one or the other of these factors is greatly reduced

When whole blood is placed in silicone-coated tubes the initial delay is prolonged, but the explosive liberation of thrombin when it occurs is normal The delay phase can be shortened to normal by the addition of thrombin or serum

If it be assumed that the explosive phase of thrombin formation is initiated by the appearance of a blood thromboplastin system, then a number of facts about this system can be summarized The antihaemophilic globulin and platelets are necessary for the development of this thromboplastin The normal delay in thrombin formation is absent or much less marked in thrombocytopenic or haemophilic serum both of which contain an excess of prothrombin Serum and thrombin both shorten the delay phase which precedes the explosive liberation of thrombin The delay phase is lengthened if the clotting of normal blood occurs in silicone-coated containers

THE POTENCY OF PLASMA THROMBOPLASTIN

Using whole untreated normal plasma from which most of the platelets have been removed by centrifuging the activity of brain and the natural plasma thromboplastin can be compared (Fig 18) The concentration of brain extract used clotted normal recalcified citrated plasma in thirty-five seconds This concentration of brain was required to ensure that all the prothrombin was converted to thrombin during coagulation A comparable conversion of prothrombin was achieved with 33 000 platelets per cu mm, and yet with this concentration of platelets the clotting time of whole blood was about four minutes Similarly in haemophilic blood, in which the intrinsic thromboplastin activity is absent high concentrations of brain extract are necessary for the complete conversion of prothrombin to thrombin (Pavlovsky et al 1949 Alexander and de Vries 1949a) Comparing the two curves in Fig 18 it is clear that the thromboplastic activity initiated by the platelets is comparable to or even more powerful than, that caused by brain extract.

These experiments naturally throw doubt on the usual conception

Similar experiments can be made with normal plasma. But in normal plasma thrombin is formed and the results are necessarily less convincing because the coagulant effects of thrombin and thromboplastin cannot be distinguished From Table 16 it is clear that active thromboplastin is formed because there is no correspondence between the fibrinogen clotting times and the clotting times of the recalcified plasma

Jacox and Bays (1949) and Cazal and Izarn (1950b) made experiments on the thrombin effect of serum. They concluded that the clotting of plasma by fresh serum could not be correlated with the amount of thrombin in the serum but must be due to some other factor which appeared during clotting It is probable that these observations depend on the presence of active thromboplastin in the fresh serum

The experiments with the prothrombin deficient plasma suggest a method by which normal plasma thromboplastin activity may be studied It is necessary to prepare the essential reagents from normal blood free from prothrombin and to test these by their ability after reacting together to clot recalcified citrated plasma The first problem is naturally to discover the appropriate reagents and to free these from prothrombin Previous work suggests that both platelets and antihaemophilic globulin are required. These two reagents can be provided free from prothrombin by using $Al(OH)_3$ treated plasma (which contains antihaemophilic globulin) and washed platelets When a mixture is made of these two reagents and $CaCl_2$ and the mixture is incubated at 37 C little thromboplastic activity is formed (Table 17) Clearly some other reagent or reagents are required. The fact that Factor VII is required for the activation of brain extracts suggested that this factor might also be necessary for plasma thromboplastin formation Factor VII is adsorbed by $Al(OH)_3$ but present, relatively free from prothrombin in normal serum. A successful attempt was then made to form thromboplastin in a mixture of $Al(OH)_3$ treated normal plasma normal serum platelets and $CaCl_2$ (Table 17) From Table 17 it will be seen that mixtures omitting one or other of the reagents gave long clotting times indicating that all three reagents are required for thromboplastin formation.

Though thromboplastin from plasma constituents is unstable it retains its activity for long enough to test the effects of dilution The potency of plasma thromboplastin is more rapidly reduced by dilution than is that of brain extract Most of its activity disappears

appeared Whether it is neutralized by an inhibitor or is intrinsically unstable is not known It has not yet been isolated from plasma in a pure form

TABLE 15

THE FORMATION OF THROMBOPLASTIN IN PROTHROMBIN DEFICIENT PLASMA

0 3 ml of ½ plasma were mixed with 0 3 ml. platelets suspension (313 000 per c mm) and 0 3 ml of M/40 $CaCl_2$ At intervals 0 1 ml. of this incubation mixture was added to 0 1 ml of normal plasma and the mixture immediately recalcified with 0 1 ml. of M/40 $CaCl_2$

The clotting times are recorded in seconds The fibrinogen clotting times give an indication of the amounts of thrombin transferred from the incubation mixture

Incubation mixture	*Substrate*	*Time intervals in minutes for withdrawal of samples from the incubation mixture*								
		½	1	2	3	4	6	8	16	32
Equal parts of (1) Prothrombin deficient plasma diluted ½	Recalcified normal plasma	37	24	18	16	16	15	15	17	21
(2) Platelets (3) M/40 $CaCl_2$	Fibrinogen	660	163	300	460	370	960	1800+		

TABLE 16

THE FORMATION OF THROMBOPLASTIN IN NORMAL PLASMA

The experiment was performed in the same way as was that recorded in Table 15 The clotting times are recorded in seconds

Incubation mixture	*Substrate*	*Time intervals in minutes for the withdrawal of samples from the incubation mixture*					
		1	2	4	8	16	32
Equal parts of (1) Normal plasma diluted ½	Recalcified normal plasma	47	10	10	13	16	27
(2) Platelets (3) M/40 CaCl	Fibrinogen	120	30	25	100	300+	300+

of the belief that the physiological conversion of prothrombin depends mainly on a supply of tissue extract and secondly it makes possible an effective study of patients in whose blood thromboplastin formation is deficient The demonstration of thromboplastic activity is the essential first step which leads to the study of the reactions by which it is formed.

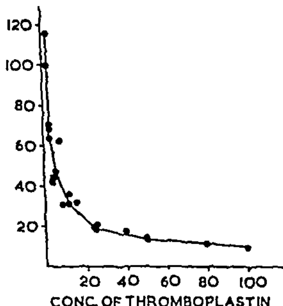


Fig 19 Varying dilutions of plasma thromboplastin were made 0 1 ml. of each dilution was added to 0 1 ml of platelet poor plasma and the mixture was recalcified with 0 1 ml. M/40 of calcium chloride The clotting times are plotted against the concentration of thromboplastin taking the most potent thromboplastin preparation as 100 per cent The results are from experiments with several different preparations of plasma thromboplastin

In the experiments to be described the results are expressed in terms of the amount of thromboplastin formed using the curve illustrated in Fig 19 to interpret clotting times as thromboplastin concentrations A start can be made on the study of the nature of the reactions by observing the effect of diluting each of the four primary constituents (Al(OH) treated plasma, serum platelets and $CaCl_2$) The results of the experiment are shown in Figs 20 21 and 22

TABLE 17

THE FORMATION OF PLASMA THROMBOPLASTIN FROM PROTHROMBIN FREE REAGENTS PREPARED FROM NORMAL BLOOD

The reagents used in this experiment were
1 $Al(OH)_3$ treated normal plasma diluted 1 in 5 with 0 85% NaCl
2 Platelets 3 × concentrated from plasma.
3 Normal serum diluted 1 in 10 with 0 85% saline
4 M/40 $CaCl_2$
0 2 ml amounts of each of a selection of the above reagents were mixed together and incubated at 37 C at one minute intervals 0 1 ml. amounts were added together with 0 1 ml of M/40 $CaCl_2$ to 0 1 ml amounts of normal citrated plasma
The clotting times were recorded in seconds

Incubation Mixture ml					*Time in minutes at which sample is withdrawn from incubation mixture*				
$Al(OH)_3$ Plasma $\frac{1}{5}$	*Serum $\frac{1}{10}$*	*Platelets*	*0 85% Saline*	*$CaCl_2$ M/40*	1	2	3	4	8
					Clotting time in seconds				
0 2	—	0 2	0 2	0 2	65	60	57	44	44
—	0 2	0 2	0 2	0 2	64	60	55	38	33
0 2	0 2	—	0 2	0 2	120	100	65	37	45
0 2	0 2	0 2	—	0 2	60	40	18	16	14

if the preparation is diluted 1/100 whereas potent brain preparations have some thromboplastic activity when diluted 1/10 000 A dilution curve of thromboplastic activity can be made by plotting the clotting time of the plasma against the relative concentration of thromboplastin (Fig 19) Using this dilution curve it is then possible to make a rough quantitative measure of the amount of thromboplastin formed in a particular reacting mixture This use of the thromboplastin dilution curve is similar to the use of the thrombin-fibrinogen dilution curve for the measurement of thrombin (Chapters II and III) Curves for the generation of plasma thromboplastin can be made and the effects of the various factors on the formation of plasma thromboplastin can be studied

EFFECT OF VARYING EACH OF THE THREE CRUDE CONSTITUENTS NECESSARY FOR PLASMA THROMBOPLASTIN FORMATION

The observation that a powerful coagulant can be formed from three crude blood constituents is important firstly because it disposes

factors which both accelerate and inhibit clotting Using fractions of normal plasma and blood samples from patients deficient in various factors some idea of the essential components can be obtained.

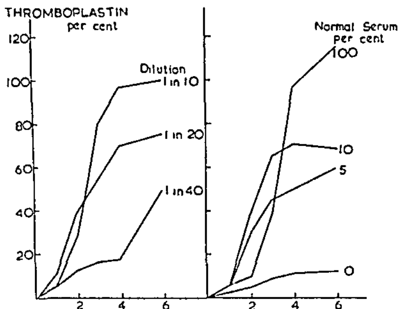


Fig 21 The curves in the left-hand diagram represent the results of the thromboplastin generation test carried out with wholly normal reagents. The platelets $Al(OH)$ treated plasma and $CaCl_2$ were at constant concentration throughout. The serum was used in saline dilutions of 1 in 10 1 in 20 and 1 in 40 in different experiments.

The curves in the right-hand diagram are derived from similar experiments in which a 1 in 10 saline dilution of normal serum was mixed in various proportions with a 1 in 10 dilution of serum from a patient with Christmas disease. The 10 per cent mixture consisted of 9 parts of diluted Christmas disease serum and 1 part of diluted normal serum.

$Al(OH)_3$ treated Plasma Plasma treated with $Al(OH)_3$ lacks prothrombin Factor VII and the Christmas factor (further reference to which will follow) and is known to contain antihaemophilic globulin and Factor V Factor V and antihaemophilic globulin (A H G) can be separated from each other by $(NH_4)_2SO_4$ fractionation the A H G being precipitated at 33 per cent saturation and the Factor V between 33 and 50 per cent saturation The omission of the A H G fraction prepared from $Al(OH)_3$ treated normal plasma Factor V being included, greatly depresses thromboplastin formation The

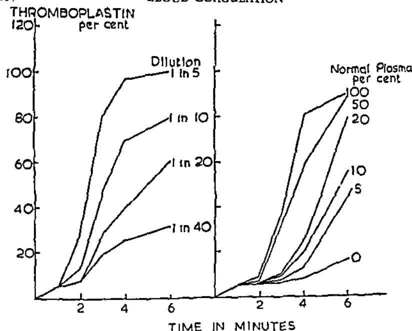


Fig 20 The curves on the left hand diagram represent the results of the thromboplastin generation test using wholly normal reagents The platelets serum platelets and $CaCl_2$ were at constant concentration throughout. The $Al(OH)_3$ treated plasma was used in dilutions with 0 85 per cent saline of 1 in 5 1 in 10 1 in 20 and 1 in 40 in different experiments The curves in the right hand diagram are derived from similar experiments in which a 1 in 5 saline dilution of normal plasma was mixed in various proportions with a 1 in 5 dilution of haemophilic $Al(OH)_3$ treated plasma The 50 per cent mixture consisted of 1 part of normal diluted $Al(OH)_3$ plasma and 1 part of diluted haemophilic $Al(OH)_3$ treated plasma

It will be seen that dilution of each of the constituents leads to an apparent reduction in the amount of thromboplastin formed Reducing the concentration of $Al(OH)_3$ treated plasma and of $CaCl_2$ also appears to delay thromboplastin formation Reduction of serum and platelets has no such delaying effect No definite conclusions about the quantitative utilization of factors can be drawn from these experiments because both serum and $Al(OH)_3$ treated plasma contain inhibitory factors and in the presence of inhibitors delay will cause a reduced amount of thromboplastin formation

WHAT FACTORS ARE CONCERNED IN BLOOD THROMBOPLASTIN FORMATION?

The reagents used to demonstrate thromboplastin formation are all crude blood derivatives each known to contain several important

mophilic $Al(OH)_3$ treated plasma is substituted for the normal sample thromboplastin formation is greatly depressed When the $Al(OH)_3$ treated plasma from a Factor V deficient patient is substituted for the normal reagent there is some depression of thromboplastin formation but less than is usual in haemophilia (Bergsagel 1953 personal communication quoted by Bergsagel 1955b)

Antihaemophilic globulin is a labile coagulation factor which deteriorates on storage and is readily destroyed by heating it is consumed during normal clotting It is precipitated from normal plasma by 33 per cent saturation with $(NH_4)_2SO_4$ and is present in Cohn s fraction I and in the beta globulin as demonstrated by electrophoresis (Hardisty and Pinniger 1956) It is not adsorbed by $Al(OH)_3$ $BaSO_4$ etc. The properties of Factor V are considered in Chapter V

Platelets Platelets are essential for thromboplastin formation but platelets are also complex having Factor V activity an accelerator for the thrombin-fibrinogen reaction a neutralizer of heparin and a thromboplastin factor (Van Creveld and Paulssen 1951 1952 and 1953 Deutsch et al 1954) The thromboplastin factor appears to occur in fraction 3 differentiated by Van Creveld and Paulssen It is heat stable The platelet factor is probably a lipoid or lipoprotein because platelet activity in thromboplastin generation can be replaced by a brain phospholipid (Bidwell and Biggs 1953 Bell and Alton 1954) The phospholipid appears to be attached to the cephalin fraction the lecithin fraction usually being inactive or inhibitory The crude cephalin fractions have a definite optimum range of activity too much being inhibitory Attempts at further purification of the cephalin by making the five fractions of Folch (1942) have given conflicting results the highest activity has usually been obtained in fraction 4 The phosphatidyl ethanolamine fraction (fraction 5) with which the Russell s viper venom potentiating action of lipoid is usually associated is relatively less active in the thromboplastin generation test

Serum Serum is a complex and unphysiological reagent it contains the products of clotting which may bear little relation to any substance present in blood before coagulation starts Serum is already said to contain 6 coagulation factors Factor VII (Koller Loeliger and Duckert 1951 1952) Christmas factor (Biggs et al 1952 Aggeler et al 1952) P T A factor (Rosenthal 1954) and Factor X (Koller 1954 1955) component D (Spaet and Aggeler 1954)

omission of Factor V has a similar but less marked effect It is clear that A H G is an essential factor for thromboplastin formation but the role of Factor V is less clear In all of the experiments platelets are used and platelets are appreciably contaminated with adsorbed Factor V (Ware, Fahey and Seegers, 1948 Hjort Rapaport and

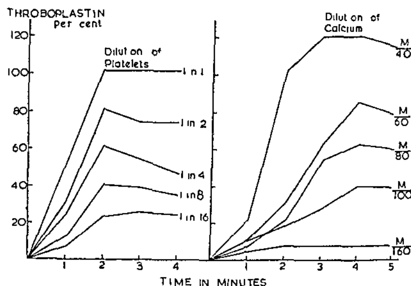


Fig 22 The curves in the left hand diagram represent the results of the thromboplastin generation test carried out with wholly normal reagents The $Al(OH)_3$ treated plasma, serum and $CaCl_2$ were at constant concentration throughout. The platelets were used undiluted and at dilutions made with 0 85 per cent saline of 1 in 2 1 in 4 1 in 8 and 1 in 16
The curves in the right hand diagram were derived from similar experiments in which the $Al(OH)_3$ treated plasma serum and platelets were at constant concentration throughout and in different experiments the concentrations of $CaCl_2$ used were M/40 M/60 M/80 M/100 M/160 (The results used for this figure were kindly supplied by Dr D E Bergsagel.)

Owren 1955) The platelet activity required for thromboplastin formation can be supplied by a phospholipid preparation made from human brain (Bidwell and Biggs 1953 Bell and Alton 1954) which contains no Factor V If this lipoid is used the omission of Factor V from the mixture causes a greater depression of thromboplastin formation than when platelets are used This evidence appears to show that Factor V may also be necessary for thromboplastin formation though possibly in quite low concentrations

The problem can also be studied using the blood of patients who lack either antihaemophilic globulin or Factor V When hae-

give reduced thromboplastin formation when used to replace normal serum. It was concluded that Factor VII was also essential But doubt has been thrown on this view by two observations In the first it appears that the defect caused by the dicoumarin anticoagulants may be very complex Not only Factor VII but also the Christmas factor and possibly also a new factor called Factor X may be involved. Deficiency in the Christmas factor was demonstrated by showing that mixtures of Christmas disease serum and serum from anticoagulant cases did not always give normal thromboplastin formation which they should had Factor VII been provided by the Christmas disease serum and the Christmas factor by the anticoagulant serum (Biggs 1954) Since it is known that the Christmas disease serum does provide Factor VII it must be presumed that the anticoagulant serum does not provide the Christmas factor The thromboplastin defect in the sera of anticoagulant treated patients cannot therefore be attributed wholly to Factor VII deficiency Moreover thromboplastin formation has now been tested in constituents derived wholly from the blood of four patients with congenital Factor VII deficiency (Hicks 1955 Ackroyd 1956 and Jurgens 1955 and Owren Newcomb Hjort 1955) In all of these cases it was found that the serum behaved entirely normally in the thromboplastin generation test A set of results kindly supplied by Hicks (Table 18) illustrates this point From this table it will be seen that not only does the thromboplastin formed clot normal plasma in the normal

TABLE 18

THROMBOPLASTIN FORMATION IN THE BLOOD OF A FACTOR VII DEFICIENT PATIENT
(Figures supplied by Hicks)

Incubation Mixture	*Substrate*	*Time of Incubation Minutes*	*Clotting time Seconds*
$Al(OH)_3$ Plasma of patient dil ⅓	Patient's Plasma	1	25 24
Patient's Platelets		2	12, 12½
		3	9 9
Patient's Serum dil. ⅒		4	8½ 8
		5	10 10
$CaCl_2$ M/40		6	10 10½

a platelet lipoid factor (O Brien 1955) and when fresh it may contain labile products of clotting (thrombin thromboplastin or intermediate products of thromboplastin formation) The presence of these may confuse the results and to eliminate them the sera to be tested should be incubated for at least 24 hours at 37° C In spite of these difficulties serum has proved practically to be a useful reagent

Serum collected after incubation at 37° C for 24 hours contains two important stable coagulation factors Factor VII and the Christmas factor which have been studied in some detail Factor VII is deficient in the blood of patients with a variety of clotting disorders including patients treated with anticoagulants of the dicoumarin type Christmas factor (Plasma thromboplastin component or haemophilic factor B) is deficient in patients with Christmas disease a condition closely resembling haemophilia which will be discussed in detail in Chapter XV

The Christmas factor can be prepared from serum free from Factor VII by the method described by White Aggeler and Glendenning (1953) which depends on the fact that the Christmas factor is very resistant to acid pH while Factor VII is destroyed Using the Christmas factor prepared in this way to replace serum thromboplastin formation appears to progress almost normally even in the absence of Factor VII (Spurling and King 1954)

The problem can be approached by using the blood of patients with clotting defects When the serum of a patient with Christmas disease is used to replace normal serum the amount of thromboplastin formed is much reduced When mixtures of normal and Christmas disease serum are used the amount of thromboplastin formed is reduced, but there appears to be an increase in the speed of thromboplastin formation (see Fig 21) All experiments suggest that the Christmas factor is involved in thromboplastin formation The Christmas factor does not deteriorate on storage is present in serum and therefore not consumed during clotting It is adsorbed by $Al(OH)_3$ $BaSO_4$ etc and is a beta globulin It is precipitated at 33 to 50 per cent saturation with $(NH_4)_2SO_4$ and is in Cohn's fraction IV

Serum also contains Factor VII (proconvertin) which has been shown to be essential for the action of tissue extracts This factor also might be necessary for blood thromboplastin formation The serum from patients treated with tromexan which lacks Factor VII was tested by Biggs Douglas and Macfarlane (1953a) and found to

cause other workers have not confirmed all of Koller's results Bergsagel (1955b) and Verstraete and Vandenbroucke (1955) and Biggs (1954) have not found complete correction of thromboplastin formation using mixtures of anticoagulant and Christmas disease sera The different workers have used sera from patients treated with a variety of anticoagulants including tromexan dindevan and marcoumar and as Koller (1955) has pointed out there may be differences in the mode of action of these drugs tromexan for instance causing a reduction in Christmas factor whereas marcoumar (used by Koller) causes a reduction in this factor only after prolonged treatment Since Factor X is labile on storage it is also of course necessary to be certain that it has not deteriorated in the Christmas disease serum used. Since the specimens used in experiments are usually stored this possibility is difficult to exclude Bergsagel has also found abnormal thromboplastin formation in mixtures of sera from cirrhosis and Christmas disease patients Bergsagel (1955) has claimed that the delayed thromboplastin formation in stored serum may be due to the dissociation on storage of an intermediate product of thromboplastin formation which is present in fresh serum.

The status of Factor X is in some doubt. Recent unpublished experiments of our own seem to confirm Koller's observation that the pattern of thromboplastin formation in patients treated with the dicoumarin anticoagulants differs from that in patients with Factor VII deficiency or Christmas disease and that the phenomenon cannot at present be attributed to any previously recognized deficiency A careful study of patients treated with different varieties of the dicoumarin anticoagulants would undoubtedly be very helpful in solving the difficulty At this stage no physiological experiments on the role of Factor X are available and so the factor cannot be included as an established factor in blood coagulation theory Evidence about this factor rests entirely on mixture experiments on the blood of patients For clarity it is easier to consider the experimental phenomenon and to refer to the Factor X phenomenon or the dicoumarin delay defect rather than to accept the true existence of a single factor controlling this abnormality

Plasma Thromboplastin Antecedent Rosenthal et al (1953) and Rosenthal (1954a and b) have described a haemorrhagic state resembling haemophilia usually with mild symptoms and with a dominant type of inheritance From both clinical and laboratory points of view the disease differs from either haemophilia or Christ-

time it also clots the patient's own plasma normally Moreover Verstraete and Vandenbroucke (1955) and Koller (1955) have now shown that abnormal thromboplastin formation may occur in the serum of anticoagulant treated cases when the Factor VII content of their blood is normal All of these observations suggest that the thromboplastin defect of the anticoagulant cases may not be due to Factor VII deficiency and that Factor VII may have little to do with blood thromboplastin formation If this last contention proves to be true there is an interesting difference between the tissue and blood thromboplastin systems the one requires Factor VII and the other does not

Factor X Koller (1954 1955) has postulated that an additional factor concerned in normal blood thromboplastin formation is present in serum Koller observed that the pattern of thromboplastin formation when Christmas disease serum is substituted for normal serum is different from that when serum from a patient receiving anticoagulant therapy is used Christmas disease serum causes a marked reduction in the amount of thromboplastin formed whereas the anticoagulant sera produce a striking delay in thromboplastin formation with little or no reduction in the final amount This difference is definite and certainly suggests that the defects are not the same Koller then claims that a mixture of Christmas disease and anticoagulant serum produces normal thromboplastin formation thus confirming the existence of two factors Since Factor VII is now not thought to be an essential blood thromboplastin component (see previous section) a new factor Factor X, is proposed Factor X can be prepared free from Factor VII and the Christmas factor by $BaSO_4$ adsorption of normal serum in the presence of citrate Factor X differs from Factor VII and the Christmas factor in being labile on storage and therefore absent from stored serum The purified Factor X will correct the abnormality of the anticoagulant sera but not that of the Christmas disease patients Stored normal serum will correct the abnormality of Christmas disease serum but not that of the anti-coagulant treated cases

Koller has also claimed that Factor X is deficient in the serum from patients with cirrhosis of the liver thus mixtures of Christmas disease serum and serum from cirrhosis patients produces normal thromboplastin formation when other normal reagents are present

The case for the existence of Factor X seems very complete from Koller s observations but unfortunately the picture is confused be-

Christmas disease and P T A deficient samples These experiments are not entirely conclusive Patients with combined deficiencies might have a relatively high level of Christmas factor and antihaemophilic globulin and 1 per cent of either of these factors produces a normal clotting time Thus if the patient s blood contained 20 per cent of A H G a 1 in 10 mixture with haemophilic blood would provide 2 per cent of A H G in the mixture and a normal

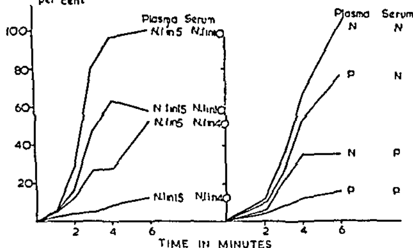


Fig 23 The curves in the right-hand diagram were derived using reagents prepared from the blood of a patient with a bleeding tendency similar to that of Rosenthal s syndrome (P T A. deficiency) The upper curve represents the control mixture with wholly normal reagents in the other experiments either the Al(OH) treated plasma or the serum or both are replaced by similar reagents from the patient as indicated. (N = Normal reagent. *P* = Patient s reagent.)

The curves in the left-hand diagr m represent results obtained using the indicated saline dilutions of wholly normal reagents. It will be seen that a pattern of results similar to those of the right-hand diagram are obtained. Moderate reduction of one reagent or the other has much less effect than simultaneous reduction of both.

clotting time Adsorbed normal serum is usually an inert substance in clotting but should theoretically correct the P T A defect It is claimed by Rosenthal that the clotting defect in his patients is corrected by $BaSO_4$ treated serum but in two of our patients there was no correction. On the other hand there is no guarantee that our patients are similar to those of Rosenthal

The position at the moment is far from clear The lack of definition

mas disease and Rosenthal has claimed that the patients are deficient in a new clotting factor In investigating 33 families Rosenthal (1954b) has found 3 of these patients and Frick (1954) has found 4 among 55 patients In examining 105 families we have seen 5 families with similar dominant inheritance and atypical laboratory findings but we are very doubtful if the conditions that we have seen can be attributed with certainty to the deficiency of a new factor An attempt was made to find out if our cases were similar to those of Rosenthal by sending a sample from one of our patients to Rosenthal who said that the sample corrected his patient's clotting defect

On closer study Rosenthal s factor proves to have rather unusual features Rosenthal (1955) Ramot et al (1955) The defect is corrected in vitro by $BaSO_4$ treated plasma and serum and by eluates from $BaSO_4$ adsorbtion The factor is present in Cohn s fractions I and IV and some activity is present in the beta globulin fraction of serum Rosenthal (1955), Ramot et al (1955) find activity in some samples of Cohn's fractions and not others The factor therefore appears to separate sometimes with the antihaemophilic globulin and sometimes with the Christmas factor and sometimes with neither of these factors Rosenthal (1955) notes that storage of deficient samples leads to an increased P T A activity (not confirmed by Ramot et al 1955) if samples may improve on storage the negative results obtained by Rosenthal with our sample does not necessarily mean that our patient differs from his

Using the thromboplastin generation test a definitely abnormal pattern of thromboplastin formation is found in the P T A deficient patients (see Fig 23) These results were obtained using the blood of one of our patients and they are similar to those recorded by Ramot et al (1955) When either the normal serum or $Al(OH)_3$ treated plasma are substituted by the patient s reagents the results are nearly normal but when both of the patient s reagents are used together abnormal results are obtained These results are taken to indicate that the abnormality is due to the deficiency of a factor which is not adsorbed by $Al(OH)_3$ and present in serum the factor thus differing from previously described factors It must be emphasized that these findings have other explanations There might be a partial deficiency of both antihaemophilic globulin and the Christmas factor or some inhibitory factor may be present Ramot et al considered the possibility of a combined A H G and Christmas factor deficiency but excluded it because of mutual correction between haemophilic

TABLE 19

A COMPARISON OF THE PROPERTIES OF FACTOR V ANTIHAEMOPHILIC GLOBULIN AND CHRISTMAS FACTOR

Property	*Factor V*	*Antihaemophilic Globulin*	*Christmas Factor*
Precipitation by $(NH_4)_2SO_4$	33 50 saturation	25 33% saturation	33 50% saturation
Cohn s Fractions	III	I	IV
Ether Fractionation	?	Precipitated with fibrinogen	Precipitated with the crude alpha and beta globulin
Electrophoetic characterization	Too unstable for testing	? beta-globulin	beta-globulin
Absorption by $Al(OH)_3$ and $BaSO_4$	Not absorbed	Not absorbed	Absorbed
Storage stability	Labile	Labile	Stable
pH Stability	Labile at all pH levels	Optimum stability 6.2–7	Resists pH_3
Consumption during clotting	Consumed	Consumed	Not consumed

Bergsagel (1955a) Hougie (1955a) Bergsagel and Hougie (1956) and Bergsagel (1955b) have made valuable contributions to this subject Bergsagel observed that if decalcified serum is used in the thromboplastin generation test to replace normal untreated serum, thromboplastin formation is poor The original activity can be restored by the addition of calcium but the reactivation is slow occupying some hours If antihaemophilic globulin is present the reactivation is much accelerated Native plasma and amberlite decalcified plasma behave like decalcified serum when used to replace serum in the thromboplastin generation test These results suggest that during clotting a reaction occurs between calcium Christmas factor and antihaemophilic globulin The product of this reaction appears to be a calcium complex which is inactivated by decalcifying agents This calcium complex is present in fresh serum but as it is unstable it is absent from aged serum

of Rosenthal's 'factor , the strange increase in activity on storage and the laboratory tests on our cases make us very hesitant to accept this new factor though careful study of the patients such as those of Rosenthal and his co-workers must in the end provide a rational explanation

A possible Fourth Plasma Thromboplastin Component
Plasma thromboplastin factor D

In 1954 Spaet Aggeler and Kinsell described a patient with a clotting defect similar to that of Rosenthal's 'P T A' deficient patients but whose blood corrected the defect in one of Rosenthal s cases As little as 2 per cent of the patient s plasma would correct the abnormal prothrombin consumption of haemophilic or Christmas disease blood It must be presumed that this patient had no marked deficiency of antihaemophilic globulin or Christmas factor On the other hand the evidence that the patient differed from Rosenthal s patients is not good because it appears that matching experiments on postal samples are unreliable

To summarize it now appears that 5 factors are reasonably well established as essential for blood thromboplastin formation These are calcium, antihaemophilic globulin Christmas factor ('P T C') and a platelet factor (probably a lipoid) and Factor V (proaccelerin) The properties of Factor V antihaemophilic globulin and Christmas factor are compared in Table 19 In addition there are a number of observations attributed variously to Factor X deficiency P T A deficiency or plasma thromboplastin factor D deficiency which remain to be explained Factor VII does not appear to be essential for blood thromboplastin formation which is remarkable since this factor is required by tissue extracts If this conclusion is widely confirmed any analogies between the blood and tissue thromboplastin systems become uncertain

THE MODE OF INTERACTION OF THE THROMBOPLASTIN COMPONENTS

Owren (1955) Owren Rapaport Hjort and Aas (1954) have put forward a hypothesis of blood coagulation including the possible interactions of the thromboplastin (prothrombinase) components This hypothesis is built almost entirely on work involving tissue extracts the blood system being deduced by analogy The work suggesting that Factor VII is not necessary for blood thromboplastin formation throws much doubt on such deductions by analogy The reactions of the blood system must be determined independently

reaction can be inactivated by decalcifying reagents calcium thus presumably forms an integral part of the complex

No mention has so far been made of Factor V In the experiments of Bergsagel and Hougie (1955) Factor V was present in the platelet preparations that they used Since it is probable that Factor V is an important constituent of thromboplastin experiments in which the level of Factor V is controlled are required. Factor V may be necessary for the formation of the active platelet coagulant or this coagulant when formed may undergo a further reaction with Factor V No single hypothesis for the interaction of the thromboplastin precursor factors can yet be written but two equally possible views are reproduced below

1 Christmas factor + antihaemophilic globulin + calcium
=Intermediate product 1
Intermediate product 1 + Factor V + calcium
=Intermediate product II
Intermediate product II + Platelets + calcium
=Intermediate product III

2 Christmas factor + antihaemophilic globulin + calcium
=Intermediate product I
Intermediate product I + Platelets + calcium
=Intermediate product II
Intermediate product II + Factor V + calcium
=Intermediate product III

It is important not to be too dogmatic about these very hypothetical reactions or a ridiculous confusion may arise from product II becoming product III or product I becoming product II or any mixture having some other designation The final product of these reactions if it exists as a single substance may be a direct activator of prothrombin or a prothrombinase according to the terminology of Owren (1955) The sedimented product studied by Bergsagel and Hougie (1956) is a very powerful coagulant A similar coagulant has been prepared from human reagents by Nour-Eldin and Wilkinson (1956) using precipitation with ether in the cold There is little evidence about the mode of action of either of these substances and until this information is available we prefer to use the term plasma thromboplastin rather than prothrombinase particularly as there is some doubt about the identity of the plasma and tissue activators of prothrombin

It was then found that if this calcium complex made from ether extracted serum (ether removes any possible platelet factors that might be present O Brien 1955) were added to plasma containing platelets short clotting times (12-15 sec) were obtained whereas platelet free plasma was clotted in 40-60 sec Antihaemophilic globulin or serum alone clotted platelet containing plasma in more than 60 sec These observations support the view that some reaction occurs between calcium antihaemophilic globulin and the Christmas factor and further suggests that the product of this reaction interacts with platelets

Hougie (1955a) observed that when citrated plasma containing platelets is stored in glass tubes an increase in the coagulant activity of the platelets occurs This change is a qualitative one because simple increase in the number of platelets does not increase their coagulant ability These findings naturally suggested that some factor or factors in the plasma produced a qualitative change in the platelets

Working together Bergsagel and Hougie (1956) have found that the maximum activation of platelets occurs when they are incubated with antihaemophilic globulin the Christmas factor and calcium in other words the calcium Christmas factor complex studied by Bergsagel reacts with platelets Bergsagel (1955b and 1956) has observed the microscopic changes which occur in platelets that are in contact with Christmas factor antihaemophilic globulin and calcium The platelets agglutinate and then become swollen and finally release numerous very small granules Centrifugation studies (Bergsagel and Hougie 1956) have shown that whereas untreated platelets can be sedimented by centrifugal speeds of 3000 r p m the product of their reaction with antihaemophilic globulin Christmas factor and calcium requires to be centrifuged at 30 000 r p m for 1 hour before all of the material is deposited These observations confirm the belief of Brinkhous (1947) that antihaemophilic globulin is involved in a reaction which leads to platelet disintegration and the release of a platelet thromboplastin factor

The product of this reaction is a remarkable coagulant which when concentrated will clot platelet poor plasma in the presence of calcium in 5 seconds It is composed of a thermolabile and thermostable component the latter presumably being derived from the platelets because heat inactivated material can be reactivated by incubation with a fresh supply of antihaemophilic globulin calcium and Christmas factor The active component liberated from platelets in this

little Factor V If dilutions of formed blood thromboplastin are tested by the two-stage method it will be seen that dilution greatly affects the speed of thrombin formation (Fig 24) This experiment was carried out on a system freed from antithrombin It appears that dilution also affects the amount of thrombin formed However even in experiments which exclude inhibitors it would be unwise to deduce a quantitative reaction between prothrombin and plasma thromboplastin without much confirmatory evidence

CALCIUM AND THE FORMATION OF BLOOD THROMBOPLASTIN

Calcium appears to be essential for blood thromboplastin formation and action The work of Bergsagel (1955a and b) suggests that the early stages of thromboplastin formation calcium is incorporated into the Christmas factor molecule Decalcification at any stage removes the coagulant activity and in the final reaction with citrated plasma a further addition of calcium is always necessary if minimum clotting times are to be obtained. It therefore appears that calcium is very generally required

Bergsagel (1955a and b) considers that calcium reacts stoichiometrically with the Christmas factor because he has found that the amount of intermediate formed from these two reagents in the presence of antihaemophilic globulin is directly related to the amount of calcium present initially Since his product was considered to be free of inhibitory substances this conclusion is reasonable But while the nature of these reactions remains in doubt this cannot be accepted as proved Quick (1940) (1947d and e) also thought that calcium reacts stoichiometrically with a blood coagulation factor

THROMBIN AND THE FORMATION OF BLOOD THROMBOPLASTIN

The catalytic action of thrombin on clotting has been emphasized by Quick (1951a) Owren (1947) Ware and Seegers (1948b) Its effect on thrombin formation in whole blood has been illustrated in Fig 14 The addition of small amounts of thrombin to the reagents required for thromboplastin formation leads to a marked acceleration of thromboplastin formation (Fig 25) Quick has attributed the acceleration to a labilizing effect on the platelets Observations of Bergsagel and Hougie (1956) have not confirmed this belief Platelets treated with thrombin do not have the marked coagulant ability which they develop in the presence of Christmas factor

THE REACTIONS OF FORMED PLASMA THROMBOPLASTIN

A preliminary study by Bergsagel and Hougie (1956) has shown that the sedimented coagulant will clot plasma from haemophilic Christmas disease and Factor VII deficient subjects as rapidly as it will clot normal plasma Product III made from Factor VII deficient reagents is just as effective confirming the view that Factor VII is not required by the blood thromboplastin system Stored oxalated plasma (Factor V deficient) has a slightly longer clotting time than normal in the presence of product III' and calcium

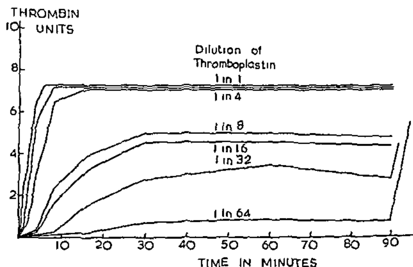


Fig 24 The curves show the results of experiments to demonstrate thrombin formation from purified prothrombin using blood thromboplastin as the activator of prothrombin conversion The blood thromboplastin was formed in a mixture of purified antihaemophilic globulin Christmas factor made by the method of White Aggeler and Glendenning (1953) Factor V freed from antithrombin by $(NH_4)_2SO_4$ precipitation washed platelets and CaCl *These reagents were allowed to react together until maximum activation was reached when* the mixture was placed on ice where it remained stable for several hours Saline dilutions of this plasma thromboplastin mixture were made

Thrombin formation was tested in mixtures consisting of equal parts of purified prothrombin, plasma thromboplastin in various dilutions and M/40 $CaCl_2$ The thrombin *activity was tested on a substrate of purified fibrinogen At 90 min. concentrated thrombo*plastin was added to the mixtures containing the least initial amount of thromboplastin and further thrombin formation could be demonstrated

When formed blood thromboplastin is added to a mixture of prothrombin and fibrinogen it clots the mixture very rapidly suggesting that it is capable of converting prothrombin to thrombin The prothrombin used in the experiment was certainly contaminated with the Christmas factor and Factor VII but contained very

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and antihaemophilic globulin Owren (1947) and Ware and Seegers (1948a) on the other hand hold that thrombin increases the activity of Factor V and their combined experiments are more convincing though further evidence is still required

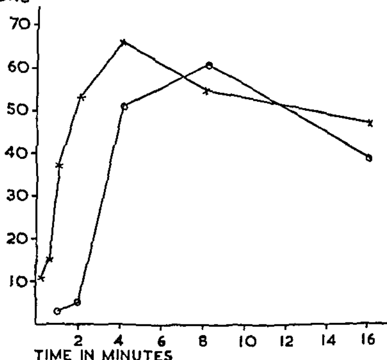


Fig 25 The formation of thromboplastin from platelets Factor VII the antihaemophilic globulin and calcium chloride in the presence (x—x) and absence (O—O) of thrombin.

THE EFFECT OF SURFACE CONTACT ON BLOOD COAGULATION

Blood circulating in the vessels contains all of the reagents required for thromboplastin formation and yet under normal circumstances clotting does not occur On contact with a glass tube clotting is initiated while clotting also occurs in silicone or paraffin-coated containers it is much delayed Several observations have been made about the effects of contact with glass

Bird blood freed from cells comparable to platelets will not clot on contact with glass If platelets are removed from very carefully

collected mammalian plasma by high speed centrifuging incoagulable plasma which will not clot on contact with glass can be obtained Jacques Fidlar Felsted and MacDonald (1946) Brinkhous (1947) Patton Ware and Seegers (1948) Buckwalter Blythe and Brinkhous (1949) It should be noted that although possible it is extremely difficult to prepare cell-free incoagulable mammalian plasma It appears that glass contact has some effect on platelets a supposition which has been supported by Biggs Douglas and Macfarlane (1953c)

Contact with glass also affects some factor in plasma Biggs Douglas and Macfarlane (1953c) provided evidence of some change in the Christmas factor Owren Rapaport Hjort and Aas (1954) confirmed this finding and also suggested that Factor VII in plasma is activated by glass contact

Thus there are three possible observed effects of contact with a glass surface It is possible that both Factor VII and the Christmas factor as they exist in serum are not normal blood constituents but are derived from inactive or less active precursors The incoagulability of circulating blood may be due to the inactive state of certain essential thromboplastin components

THE THROMBOPLASTIN INHIBITORS

Plasma thromboplastin formed from Al(OH) treated plasma serum platelets and calcium is unstable the activity gradually decreases on prolonged incubation at 37 C On the other hand the coagulant formed from partially purified reagents is stable This difference suggests that the crude reagents contain inhibitory substances which are removed from the more purified preparations The product studied by Bergsagel (1955a and b) which is formed by incubating the Christmas factor calcium and antihaemophilic globulin is also unstable and may be neutralized by some inhibitory substances Tocantins and Carroll (1949) have claimed that a thromboplastin inhibitor occurs in normal blood and is present in excess in haemophilic blood. At present nothing is known about any such naturally occurring inhibitory substance or substances but the existence of one or more inhibitors of the thromboplastin system is probable

SUMMARY TO CHAPTER VI

From a study of thrombin formation in whole blood and plasma it can be deduced that blood must contain all that is required to form a powerful thromboplastin Using the blood of a prothrombin deficient patient this thromboplastic activity was demonstrated Following this work it was possible to devise a technique, called the thromboplastin generation test, by which normal blood thromboplastin formation may be demonstrated

An attempt to define the factors which take part in thromboplastin formation has suggested that antihaemophilic globulin the Christmas factor a lipoid Factor V and Calcium are essential Factor VII is of doubtful importance Other factors suggested (plasma thromboplastin antecedent Factor X and plasma thromboplastin component D) are not yet sufficiently defined to be included

A study of the reactions which precede the appearance of thromboplastin suggests that antihaemophilic globulin and the Christmas factor undergo some reaction in the presence of $CaCl_2$ and that the product causes platelet metaphorphosis clumping and the liberation of small particles of high thromboplastic activity The final product reacts with prothrombin to form thrombin the nature of this final reaction is not yet clear

CHAPTER VII

THE NATURAL INHIBITORS OF BLOOD COAGULATION

Though blood clots readily when withdrawn from the body or when it is extravasated into damaged tissues in the vessels it remains fluid. In the living body there must be therefore an effective mechanism for suppressing coagulation Blood coagulation may be hindered by a delay in the formation of activating factors or by neutralization of active substances when formed In Chapter VI it has been suggested that the formation of plasma thromboplastin is a time-consuming reaction which depends on contact with foreign surfaces This natural delay in the formation of an essential activator may be a very important factor in preventing intravascular clotting In addition Tocantins (1943 1944 Tocantins and Carrol 1949a and b etc.) has postulated an antithromboplastic substance in blood which may be important. In normal blood there is also an efficient system for neutralizing thrombin In this chapter the antithrombin system and the antithromboplastin of Tocantins will be discussed.

Of the naturally occurring inhibitors the mechanism for the neutralization of thrombin (the antithrombin system) and heparin have been studied most widely and this problem will be discussed first

ANTITHROMBIN

Schmidt (1892) Morawitz (1905) Weymouth (1913) and Gasser (1917) were aware that thrombin disappeared rapidly from the blood after coagulation. This disappearance of thrombin was said to be due to its neutralization by antithrombin

The mechanism for neutralizing thrombin in whole plasma must be very powerful According to the work of Warner Brinkhous and Smith (1936) normal plasma contains about 300 units/ml of prothrombin If this 300 units of prothrombin were converted to 300 units of thrombin it would by definition be sufficient to clot 300 ml of fibrinogen in 15 seconds Thus the amount of thrombin formed from 10 ml of plasma would be more than enough to clot all of the blood in the body were its action unopposed

When whole blood clots in a glass tube the whole of this vast excess of prothrombin is presumably converted to thrombin since negligible amounts of prothrombin remain in the serum When the generation of thrombin is followed in whole blood there is a delay in thrombin formation lasting 3-4 minutes during which very little thrombin is formed Thereafter there is an explosive liberation of thrombin which lasts 2-3 minutes during which all of the prothrombin is converted to thrombin Yet although 300 units of prothrombin are converted to thrombin it is seldom that a level of more than 8 or 10 units of thrombin can be detected at any one time Presumably the thrombin is neutralized so rapidly that most of it is never detectable in a free form If thrombin formation is greatly accelerated by the addition of thromboplastin thrombin levels of 50 units/ml are found but this value is maintained only for a few seconds

The neutralization of thrombin in plasma can be studied by the addition of purified thrombin to plasma When this is done it is found that the ability of plasma to neutralize thrombin is very great 1 ml of plasma is capable of neutralizing about 2000 units of thrombin i e the amount of thrombin that would clot 2000 ml of fibrinogen in 15 seconds

PROPERTIES OF ANTITHROMBIN

When plasma is fractionated with ammonium sulphate or by acidification the antithrombin is separated in the albumin fraction By electrophoretic separation Lyttleton (1950) has shown that the antithrombin can be concentrated with the α-globulin fraction which contaminates the crude albumin fraction Antithrombin is destroyed by the ether method of plasma fractionation (Keckwick and Mackay 1946 1949) by chloroform by heating to 56° C and at pH values above 9 5 and below 6

THE MODE OF ACTION OF ANTITHROMBIN

In studying the mode of action of antithrombin whole plasma defibrinated plasma or serum may be used as a source of antithrombin When whole plasma is used the antithrombin activity is greater than when defibrinated plasma or serum are used This difference is due partly to the fact that thrombin is adsorbed by fibrin Thus in whole plasma there are two separate mechanisms for the removal of thrombin The removal of thrombin by fibrin is an immediate reaction If an excess of thrombin is used this immediate

reaction is followed by a progressive inactivation of thrombin by a substance antithrombin in the albumin fraction of plasma.

The immediate inactivation of thrombin due to its adsorbtion on to fibrin has been studied by Quick and Favre-Gilly (1949a) and Klein and Seegers (1950) This immediate inactivation does not occur in defibrinated plasma and can be demonstrated when thrombin coagulates isolated fibrinogen The amount of thrombin adsorbed depends on the initial concentration of thrombin With concentrations of thrombin up to 1000 units/ml 85-90 per cent of the added thrombin is removed by adsorbtion From the work of Klein and Seegers it appears that 1500-2000 units of thrombin can be adsorbed by the fibrin from 1 ml of plasma At first sight it might seem that this mechanism should explain the failure to demonstrate more than 8-10 units of thrombin during the spontaneous clotting of whole blood If 90 per cent of the thrombin were adsorbed by the fibrin then naturally it would never be possible to demonstrate more than 10 per cent of the total available thrombin Unfortunately this simple explanation is untenable because if the blood is rapidly defibrinated with a small amount of thrombin subsequent thrombin formation follows much the same pattern in the defibrinated blood as in the whole blood.

The powerful adsorbtion of thrombin by fibrin provides an excellent mechanism for preventing the dangerous extension of a thrombus If thrombosis occurs in a small vessel much of the thrombin formed may remain localized in the clot

The reaction between thrombin and antithrombin has been studied by Astrup and Darling (1942 1943) Lyttleton (1950) and Klein and Seegers (1950) Astrup and Darling found that using certain concentrations of plasma and thrombin the neutralization of thrombin by antithrombin appeared to be a first order reaction Lyttleton (1950) on the other hand found that this reaction could not be interpreted in terms of a simple first order interaction though if different amounts of thrombin were added to defibrinated plasma a constant proportion of the thrombin was neutralized in a fixed time It appears that thrombin and antithrombin react together stoichiometrically Klein and Seegers (1950) have shown that the antithrombin can be saturated by the addition of an excess of thrombin and that with amounts of thrombin less than the saturating amount the residual antithrombin is inversely proportional to the amount of thrombin added 1 ml of defibrinated plasma is capable

of neutralizing about 700 units of thrombin When more than 700 units of thrombin are added to 1 ml of defibrinated plasma the neutralization reaction proceeds rapidly and may be complete in 10-15 minutes leaving residual thrombin When a very small amount of thrombin is added to plasma it is neutralized rapidly leaving much antithrombin When the amount of thrombin added is comparable to the antithrombin content of the plasma the neutralization process may continue slowly for several hours

The careful quantitative experiments of Klein and Seegers (1950) *disclose difficulties in the interpretation of the normal coagulation* process From experiments on diluted plasma it is concluded that plasma contains about 300 units of prothrombin per ml Since one unit of prothrombin is converted into one unit of thrombin it follows that if all of the prothrombin is converted to thrombin 300 units of thrombin must be neutralized in the plasma Klein and Seegers (1950) have shown that when 300 units of thrombin are added to plasma several hours elapse before all of this thrombin disappears When whole blood clots no thrombin can be detected after 12-20 minutes

There is at present no satisfactory solution to this problem It is possible that experiments on diluted plasma may give a false impression of the amount of prothrombin present in plasma Alternatively experiments in which thrombin is added to plasma containing prothrombin may bear little relation to experiments *in which* thrombin is generated in plasma Possibly the process of prothrombin conversion activates the antithrombin system in plasma

THE MEASUREMENT OF ANTITHROMBIN

Since the antithrombin system of plasma appears to consist of two separate parts the measurement of antithrombin presents difficulties It is possible to measure the extent to which thrombin is adsorbed by the fibrin the potency of the progressive antithrombin activity or a mixture of the two systems Again it is possible to measure the antithrombin activity by recording the speed of thrombin neutralization (Astrup and Darling 1942 and Lyttleton 1950) or the total capacity of the plasma to neutralize thrombin may be measured (Klein and Seegers 1950) At present there is no reason for assuming that one system for neutralizing thrombin is more important than the other an attempt should be made to measure both

Astrup and Darling (1942) have worked out a reasonably satisfactory method of measuring the relative progressive antithrombin

capacity of different samples of plasma In this method the plasma is first defibrinated and the test carried out on defibrinated plasma A sample of the defibrinated plasma is added to a large excess of thrombin The mixture is incubated at 37 C for fifteen minutes The amount of thrombin remaining at this time is recorded by removing a sample from the mixture and adding it to fibrinogen From a comparison of the clotting time of the fibrinogen with the original thrombin solution the amount of thrombin neutralized can be calculated It must be realized that it is assumed that most of the neutralization of thrombin by antithrombin is complete in fifteen minutes From the work of Klein and Seegers (1950) this assumption will not be valid unless the amount of thrombin added is very large in relation to the amount of plasma present they use 1000-2000 units per ml of plasma In Astrup and Darling's method this high proportion of antithrombin is achieved by using small amounts of plasma (see Appendix) This method has been found to give reasonable results with normal plasma but much more study is necessary before it could be expected to be a useful routine test

To assess the ability of fibrin to adsorb thrombin the test suggested by Astrup and Darling could be repeated using whole plasma instead of defibrinated plasma The difference between the results of the two tests should give a measure of the amount of thrombin adsorbed by the fibrinogen

HEPARIN

As an incidental finding in experiments designed to purify cephalin thromboplastin from tissues McLean (1916) found that a substance could be extracted which retarded coagulation This substance was called heparin because it was first isolated from liver Howell and Holt (1918) found that heparin could be injected into animals without causing any ill effects and that it prolonged the clotting time of the animal's blood. The presence of heparin in the blood prevented the conversion of prothrombin to thrombin and Howell therefore reasonably concluded that heparin was an antiprothrombin Since McLean and Howell's original work many experiments have been carried out with heparin to isolate it in a pure form, to determine the chemical formula to discover its mode of action, and to apply the anticoagulant to the treatment of patients with thrombosis An excellent summary of this work is given by Jorpes (1946)

THE ISOLATION OF HEPARIN AND ITS CHEMICAL COMPOSITION

Heparin is present in most of the tissues of the body but is present in highest concentration in the liver and lungs Heparin is soluble in water and precipitated by alcohol acetone and acid it forms an insoluble compound with brucine (Fischer and Schmitz 1933) The methods for preparing heparin usually consist in its extraction from the tissue in water alkali or potassium thiocyanate and its purification by repeated precipitations with alcohol acetone or brucine

From chemical analysis of the purest preparations Jorpes (1946) found that the substance was composed almost entirely of hexuronic acid hexosamine and ash The ash contained 7-8 per cent of sulphur The substance therefore differs from chondroitin sulphuric acid, in its content of sulphur the nature of its amino sugar (glucosamine and not galactosamine) and in its anticoagulant ability The anticoagulant activity of heparin preparations from the same tissue increases with the number of sulphur atoms per molecule of uronic acid It is probable that commercial heparin consists of a mixture of different polysulphuric esters When preparations made from different animals are compared the close correspondence between activity and sulphur content disappears Jaques and Waters (1940) and Jaques Waters and Charles (1942) prepared heparin from dogs cows pigs and sheep and although the sulphur content was similar in all the preparations they varied enormously in activity

THE PROPERTIES AND SITE OF ORIGIN OF HEPARIN

Heparin carries a strong electric charge In a 2 per cent solution the specific conductance is 85×10^{-4} mhos Heparin solutions have no osmotic effect on the red cells Heparin stains an intense blue violet with toluidine blue Holmgren and Wilander (1937) found that the granules of mast cells stain in a similar manner and the yield of heparin from a particular tissue is related to its mast cell content It is possible that the heparin in the body arises in the mast cells The common distribution of mast cells along the capillaries suggests that heparin may have some local effect in preventing capillary *thrombosis* though heparin is *seldom found* in normal blood

THE MODE OF ACTION OF HEPARIN

Initially Howell thought that heparin acted as an antiprothrombin and the observations on which this belief was based have not been contradicted When heparin is added to blood in a concentration

sufficient to prevent coagulation the prothrombin is not converted to thrombin In a system in which the albumin fraction of the plasma had been removed Mellanby (1935a) found that heparin did not prevent the conversion of prothrombin to thrombin but inhibited the thrombin-fibrinogen reaction Quick (1938) extended the findings of Mellanby when he showed that heparin required a component from the albumin fraction of plasma for its inhibitory effect Heparin by itself did not inhibit the thrombin-fibrinogen reaction but this reaction was inhibited if albumin was present Brinkhous Smith Warner and Seegers (1939) showed that heparin does not prevent the conversion of prothrombin to thrombin in the absence of the albumin fraction Heparin therefore appears to require a co-factor for its activity From the work of Snellman Sylven and Julen (1951) it is possible that heparin as it occurs in the cells may be in combination with this co-factor and that the methods of heparin preparation normally separate the compound into two parts The albumin fraction of plasma can replace the normal co-factor According to Snellman et al (1951) the co-factor is a lipoprotein

From this work it can be concluded that in the presence of its co-factor heparin delays the thrombin-fibrinogen reaction and prevents the formation of thrombin from prothrombin

The inhibitory action of heparin on the thrombin-fibrinogen reaction has been studied by Astrup and Darling (1942) Lyttleton (1950) Klein and Seegers (1950) and Snellman et al (1951) It appears that heparin has two distinct effects In the first place it promotes the adsorbtion of thrombin by fibrin (Klein and Seegers 1950) Secondly heparin in association with the albumin fraction of plasma combines with thrombin (Lyttleton 1950 Snellman et al 1951) This combination of heparin and thrombin is reversed by toluidine blue or dilution and is affected by changes in ionic strength (Lyttleton 1950) Lyttleton (1950) and Snellman et al (1951) studying the electrophoretic pattern have shown a change in the speed of migration of thrombin when heparin and its co-factor are present This combination of heparin and thrombin reduces the ability of the thrombin to clot fibrinogen. From the work of Lyttleton (1950) it appears probable that the albumin co-factor for heparin is antithrombin This problem is difficult to study because the thrombin activity of a mixture must naturally be judged by its effect on fibrinogen. If heparin is present this may interfere with the reaction

between thrombin and fibrinogen in the indicator system From experiments with very concentrated thrombin solutions which were greatly diluted to estimate the residual thrombin Klein and Seegers (1950) have shown that heparin does not increase the amount of thrombin neutralized by antithrombin or the speed of the reaction In other words heparin interferes with the thrombin-fibrinogen reaction but does not influence the neutralization of thrombin This conclusion is interesting but if as Lyttleton (1950) has claimed the combination of heparin and thrombin is reversed by dilution it is possible that Klein and Seegers' (1950) conclusion is not valid In earlier experiments Seegers et al (1942) had shown in contrast to the later work that heparin accelerated the combination between antithrombin and thrombin It seems possible that these original observations were valid and that the combination catalysed by heparin is reversed by dilution

From all these experiments it appears that in the presence of antithrombin heparin and thrombin form a reversible combination in which the thrombin is inactivated Heparin does not increase the amount of thrombin neutralized by antithrombin but may increase the speed of this combination Since the total amount of thrombin neutralized is not increased it seems likely that antithrombin enters into the combination between heparin and thrombin Heparin does increase the amount of thrombin neutralized by whole blood or plasma because it increases the amount of thrombin adsorbed by fibrin

When heparin is added to whole blood it delays or prevents the conversion of prothrombin Biggs Douglas and Macfarlane (1953) have shown that heparin inhibits the formation of blood thromboplastin This inhibition would be sufficient to account for the failure of prothrombin conversion Conley Hartmann and Morse (1949a and b) found that minute traces of heparin were sufficient to prevent the clotting of highly centrifuged normal plasma in glass tubes Presumably the removal of an essential thromboplastin component (platelets) would account for the extreme sensitivity to heparin

Heparin circulating in the blood has also been shown to activate a plasma lipase which has the effect of clearing plasma of chylomicra the fat being absorbed by the albumin fraction with which the fatty acids combine (Robinson and French 1953) This effect is produced in vivo in vitro activation requires the presence of a tissue extract It is possible that this clearing effect might be important in

the anti-thrombosis effect of heparin if thrombosis is found to be associated in any way with a circulating thromboplastic lipoid

THE MEASUREMENT OF HEPARIN

Two *main methods for the measurement of heparin* are available In one method (Jaques and Charles 1941) oxalated blood is used To this blood varying concentrations of a standard heparin preparation are added. Thrombin is then added to the mixtures In the tubes containing heparin the clotting time lengthens with increasing concentration of heparin until a point is reached where no clot forms A test preparation is compared with the standard and the concentration of heparin is read from the first tubes in which no clot forms This method is described in the appendix

The second method depends on the isolation of heparin from blood and its measurement by the development of metachromatic activity (Jaques Mitford and Ricker 1947, Monkhous Stewart and Jaques 1949) This is more elaborate than the measurement of clotting time and the two methods do not always give exactly the same results (Snellman Jensen and Sylven 1949) Since it is the inhibition of clotting which is physiologically important the first method is preferable

ANTITHROMBOPLASTIN

From work on the effects of surface contact on blood coagulation Tocantins (1944a and b 1945 1946 Tocantins and Carroll 1949a and b) has become convinced that blood contains an antithromboplastin Tocantins believes that this antithromboplastin is present in whole blood when it is withdrawn from the body and that it is removed from blood by glass surfaces or by contact with asbestos fibres Tocantins has found that when a lipoid thromboplastin (the ether soluble alcohol insoluble fraction prepared from acetone dried brain) is added to plasma the accelerating effect of this preparation is reduced by incubation of the thromboplastin with plasma The extent of the inactivation of the thromboplastin is increased if the plasma is collected with special precautions to avoid contact and incubation takes place in lustroid tubes The plasma in lustroid tubes therefore contains more antithromboplastin than that in glass tubes If the plasma is treated with asbestos the clotting time on the addition of the lipoid thromboplastin is greatly reduced and the speed of thrombin formation is correspondingly increased More-

over if plasma treated with asbestos is incubated with the thromboplastin it is found to have lost its ability to neutralize the thromboplastic activity Haemophilic plasma has a longer clotting time than normal on the addition of the lipoid thromboplastin and a greater ability than normal to neutralize thromboplastin The clotting time of haemophilic plasma can be reduced to normal by incubation with asbestos The defect in haemophilic blood is therefore according to Tocantins due to an excess of inhibitor Tocantins has extended his observations by the final and usually crucial test He has isolated an inhibitor from normal and haemophilic blood which interfered with the reaction of plasma to lipoid thromboplastin Haemophilic plasma was found to contain an excess of this inhibitory substance Tocantins et al (1951) have observed that not only is the inhibitory effect removed by contact but it is also greatly affected by dilution In the majority of experiments on blood clotting the plasma is diluted In the one-stage prothrombin test for example the plasma is diluted to about 26 per cent in the final test Tocantins et al (1951) found that undiluted plasma has a remarkable resistance to the action of thromboplastin If the one-stage prothrombin test is carried out with various dilutions of plasma in the final mixture it was found that with 70 per cent of plasma the clotting time was 27 seconds while with 26 per cent of plasma the clotting time was 17 seconds In both these tests the concentration of thromboplastin was the same

Tocantins experimental evidence is convincing and his interpretation reasonable The active thromboplastin formed from plasma gradually disappears with the passage of time (Chapter VI) and presumably it is neutralized Tocantins substance may well be responsible for this inactivation On the other hand few would agree with Tocantins that the defect in haemophilic blood is due entirely to an excess of inhibitory substance Moreover there is much evidence that surface contact liberates an activator of blood coagulation Before Tocantins hypothesis can be accepted his work must be correlated with that of other workers on the effects of surface contact on blood coagulation

A suggestion was put forward by Macfarlane (1942) who thought that plasma thromboplastin was likely to consist of a labile protein fraction probably an enzyme and a lipoid factor Together these two factors constituted thromboplastin but they were inactive separately It was also suggested that the lipoid if it was present in excessive amounts might act as an inhibitor of coagulation A very

similar view has been put forward by Overman (1949) Shinowara (1951a and b) has demonstrated that two fractions can be separated from the plasma which together have thromboplastic activity but which are inactive separately It is possible that inhibition of coagulation by an excess of natural lipoid might account for some of Tocantins observations and that the inhibitory action of haemophilic plasma might be due to a disproportion between lipoid and protein constituents from the absence of one thromboplastin component rather than to any real excess of inhibitor

SUMMARY TO CHAPTER VII

From the work discussed in this chapter there is one well established inhibitory system in the blood. This is the antithrombin system Thrombin is normally neutralized by its adsorption on to fibrin during clotting and by its quantitative reaction with a substance known as antithrombin in the albumin fraction of plasma Heparin is not normally present in blood in measurable amounts but when it appears in pathological conditions or is introduced by injection heparin enhances the normal antithrombin system of plasma Heparin increases the amount of thrombin adsorbed by fibrin and may combine with thrombin either to make the thrombin less reactive or to render it more rapidly neutralized by antithrombin The depression of thrombin formation which occurs in the presence of heparin may be due to the rapid neutralization of free thrombin which may prevent the activation of the plasma thromboplastin system.

From work on the effect of contact with glass surfaces on blood coagulation Tocantins has concluded that blood contains a substance which neutralizes thromboplastin This antithromboplastin has been isolated from normal plasma by Tocantins but much remains to be discovered about its mode of action At the present it must be regarded as a hypothetical factor because his work has not as yet proved that the substance isolated from plasma is responsible for the phenomena which occur when plasma is exposed to glass surfaces However Tocantins hypothesis explains many observations and doubtless future experiments will disclose the mode of action of the inhibitor

CLOT RETRACTION

When blood coagulation is complete the clot usually undergoes two changes contraction and, after a variable time spontaneous lysis It is not certain that these two processes are concerned with or even the remote result of the process of coagulation but since this point is in some doubt and as both changes may have some bearing on the efficiency of coagulation they will be briefly considered in this and the following chapter

THE MEASUREMENT OF CLOT RETRACTION

The fact that newly formed blood clots have a tendency to contract so that serum and a small proportion of red cells are squeezed out has been familiar observation since the days of the phlebotomists Hewson (1772) described how soon after blood jellies or coagulates it separates into two parts the crassamentum and the serum Thackrah (1819) was interested in this contraction since it seemed to vary in degree in different clinical conditions and he devised a method of measuring its extent by which the weight of the unclotted blood was compared with the weight of the blood after contraction was complete He mentions that normal contraction results in a shrinkage of the blood clot to about 42 per cent of the weight of the original blood The word retraction now commonly used to describe this process was apparently introduced by Schklarewsky in 1868 to describe the behaviour of blood allowed to coagulate in capillary tubes since the clot would be seen to shrink away from the glass walls when viewed with a microscope The term is not particularly apt but from deference to custom it has persisted Hayem (1878 1895) described a number of the factors concerned in retraction and stimulated considerable interest in the phenomenon His method of measurement was comparatively crude consisting of watching the behaviour of clotted blood at the bottom of a small test tube He showed that the platelets were probably concerned since if the platelets were allowed to sediment out of horse blood kept fluid in a segment of excised jugular vein the resulting plasma failed to produce retractile clots Since that time a number of

methods for estimating the amount and speed of retraction have been devised but few of them have been superior to that used by Thackrah Cesana (1909) used a modification of Thackrah s method suspending a tube containing a clot from a balance which recorded the change in weight as the serum drained out of the tube Opitz and Marzdorf (19-1) demonstrated that narrow tubes such as were used by Glanzmann (1918) may cause gross errors in measuring retraction since even normal blood may fail to retract under such conditions They favoured a watch glass technique which had been used previously by Morawitz and Bierich (1907) Fonio (1921) beginning an extensive investigation of retraction which has lasted for many years used a retractilometer consisting of a graduated tube coated with paraffin in which the decreasing height of a blood clot is measured on a scale Levy-Solal and Tzank (19-3) measured the volume of serum extruded by retracting blood clots a method also used with various modifications by Boyce and MacFetteridge (1937) and Macfarlane (1939) In the latter s method a graduated centrifuge tube is filled to the five ml mark with blood and a glass rod with a number of projections is placed in the tube before the blood clots One hour after coagulation the clot is removed by lifting out the glass rod The serum volume is then read off on the scale of the graduated tube All these methods of estimating retraction involved a certain amount of manipulation or trauma to the blood clot This was avoided by suspending a volume of blood in a clear bland oil medium so that retraction took place out of contact with glass or other surfaces the serum being separated and measured. Van Allen (19-7) caused the blood clot to be suspended in oil on a small platform the serum as it was extruded falling through the oil to the bottom of the tube where it could be measured on a scale Hirschboeck (1948) suspended the blood in castor oil in a test tube and noted the time at which serum was expressed. Budtz-Olsen (1951) has greatly improved this procedure by suspending a volume of blood in a mixture of liquid paraffin and trichlorethylene combined in such proportions that the blood remains suspended in the medium. Retraction is measured by removing the clot at the end of a specified time and estimating its volume by immersion in a graduated tube The serum can also be pipetted from the oil and estimated volumetrically

FACTORS INFLUENCING CLOT RETRACTION

PHYSICAL FACTORS

A variety of physical factors influence the speed and extent of retraction Babington in 1930 noticed that the shape of the vessel in which the blood clotted had an effect on the amount of serum expressed and the importance of using tubes of reasonable width was pointed out by Opitz and Matsdorf in 1921 The type of surface in contact with the blood also affects retraction although there are contradictions in the literature as to precisely what this effect may be LeSourd and Pagniez in a series of contributions (1906 1908 1913) point out the importance of the treatment of glass surfaces considering that it is essential to flame the containers immediately before use Arthus and Chapiro (1908) found that tubes coated with paraffin wax inhibited clot retraction Budtz-Olsen (1951) investigated the effect of different treatments of surfaces such as heating different forms of cleaning and coating with collodion and liquid paraffin He found that coating with collodion diminishes the amount of retraction liquid paraffin increases it and pre-heating had a variable effect The smaller the container the less the amount of retraction he observed The optimum pH lies between 6 and 8 2 (Ellicot and Conley 1951) or between 7 and 7 8 (Budtz-Olsen 1951) The effect of the temperature of the blood during retraction is particularly interesting Macfarlane (1938a) found that both the speed and extent of retraction increased with temperature up to an optimum of about 40 Above this point retraction is reduced and at 45 it is completely inhibited Even warming of the blood to 45° before clotting or retraction begins with subsequent cooling to 37 C destroys the retractile power of the clot These observations were confirmed by Budtz-Olsen (1951)

PACKED CELL VOLUME

The packed cell volume of the blood affects the extent to which retraction can occur This fact is of practical importance in any measurement of retraction in clinical conditions in which the packed cell volume may vary to any extent Macfarlane (1938a 1939) investigated this point and came to the conclusion that normal clot retraction is brought to a standstill by the physical volume of the red cells entangled in the fibrin network. After all the available serum had been squeezed out of the clot no further retraction could

occur because only a small proportion of the red cells was able to escape If the packed cell volume of the particular blood sample was known it was possible to determine whether or not retraction was complete in the sense that as much serum as possible had been extruded, and the observed retraction could therefore be corrected for variations in packed cell volume (Macfarlane 1939 Budtz Olsen 1951) Simple correction was found not to hold in cases with grossly deficient retraction since a reduction in packed cell volume then had a variable effect in increasing the amount of serum expressed. Both Macfarlane (1938a) and Budtz-Olsen (1951) decided that there was no simple method for correcting retraction estimations for variation in red cell volume just as there is no simple method for correcting the sedimentation rate for anaemia

THE PLATELETS

The most obvious intrinsic factor affecting retraction is the number of platelets Duncan (182_) long before the existence of the platelets had been recognized, observed that clot retraction was deficient in a case of purpura haemorrhagica. Hayem (1878) and Delezenne (1897) confirmed the fact that blood deprived of its platelets produced non-retractile clots It is now a familiar observation that in any clinical condition in which the platelets are reduced there is a failure of clot retraction LeSourd and Pagniez (1906 1908 1913) showed that even moderate reductions in the platelet count decreased the amount of retraction so that the two values were directly proportional to each other Macfarlane (1938a) reduced the platelet count in samples of plasma by centrifuging and estimated the retraction in mixtures of platelet containing and platelet free recalcified plasma, and confirmed that below a count of about 100 000 per c mm. this proportionality between platelets and retraction existed He observed that in clinical cases a reduction in platelets was always accompanied by a reduction in retraction although deficient retraction might occur in the presence of a normal platelet count

FIBRINOGEN AND THROMBIN CONCENTRATIONS

Despite the proportionality between the number of platelets and retraction in blood or plasma Macfarlane (1938a) found that dilution of plasma with saline did not cause a reduction of the retraction of the clots formed by subsequent recalcification although the

platelet count was of course, reduced by this procedure This apparently paradoxical observation was explained by the work of Budtz-Olsen (1951) who has shown that within fairly wide limits retraction varies directly as the platelet count and inversely as the fibrinogen content of the plasma Dilution reduces both fibrinogen and the platelet count to the same extent and the retraction therefore remains unchanged

In most instances in which a deficient retraction has occurred despite a normal platelet count there has been an underlying condition such as pneumonia Hodgkin s disease or jaundice in which high fibrinogen levels are likely to occur (Lenobre 1898 Carr and Foote 1934 Macfarlane 1938a 1939 Aggeler et al 1946) and were in fact demonstrated by Budtz-Olsen (1951) in association with deficient retraction Lundsteen (1942) considers that the optimum fibrinogen concentration for retraction is 100 mg per cent, and Quick and Hussey (1950) state that retraction is also affected by the amount of thrombin available during the process of coagulation

MECHANISM OF CLOT RETRACTION

The motive power of clot retraction has been a source of argument ever since the phenomenon itself was first investigated Even now there are still different opinions and no completely acceptable explanation has been produced Most of the various hypotheses assume that the shrinkage of the clot is due to shortening or active contraction of the fibrin fibres but there have been suggestions that by some osmotic or other physico-chemical means the serum is drawn out of the clot by the surface action of the container (Lampert 1932) The demonstration by Budtz-Olsen and others that normal contraction of clots suspended in bland solvent mixtures takes place probably disposes of this suggestion

SHRINKAGE OF FIBRIN

The most popular explanation of retraction was that newly-formed fibrin behaves like some other colloids in that it undergoes a process of shrinkage or syneresis' This is an old view more recently supported by Lampert (1932) Ebbecke (1940) and many others Ferry and Morrison (1947) do not believe that syneresis of fibrin is a likely explanation of its shortening and Budtz-Olsen (1951) points out that there are major differences

between syneresis and clot retraction The amount of syneresis plotted against time gives an S-shaped curve clot retraction a simple logarithmic one Syneresis is much less extensive than retraction and much slower requiring under the best conditions about eight days to cause a 50 per cent shrinkage of volume whereas plasma clot retraction will produce a 95 per cent reduction in volume within an hour or so Syneresis is directly proportional to the concentration of the gel retraction is inversely proportional to fibrinogen (i e gel) concentration Syneresis is only slightly sensitive to changes in temperature clot retraction is markedly so and electrolyte concentration has a large effect on syneresis but relatively little effect on retraction Finally the force exerted by gels undergoing syneresis is enormously powerful and Budtz-Olsen has shown that clot retraction is capable of exerting a pressure of only ten or twenty mms of water Nevertheless there are still suggestions that some inherent change in the fibrin fibre itself may be responsible for it undergoing a shortening process soon after coagulation Bailey Astbury and Rudall (1943) consider that fibrinogen and fibrin are members of the keratin-myosin group and have shown that there is an unstretched alpha form and a stretched beta form of both fibrinogen and fibrin Van Zandt, Hawn and Porter (1947) have studied the structure of blood clots by means of the electron microscope and have suggested that the tendency to form compound fibres might explain clot retraction but these suppositions seem to be unsupported by any direct evidence

FIBRINOLYSIS

Another explanation is that clot retraction is the first phase in the process of fibrinolysis the fibrin being shortened before being dissolved by the fibrinolytic process Roskam (1927) obtained apparent clot retraction by adding serum treated with chloroform to platelet-free plasma Chloroform serum usually contains a proteolytic enzyme and it is probable that in this experiment what was thought to be contraction of the clot was actually its partial digestion Hirose (1934) and Ferguson and Erikson (1939) are the main subscribers to this idea of pre-digestion contraction but Budtz-Olsen (1951) has shown that fibrinolysis and clot retraction are quite independent phenomena, since each can be induced without the other It is more than likely that the partial digestion of blood clot during the process of fibrinolysis has been mistaken for clot retraction by other workers

and that the loosening of the clot from the walls of the container which is brought about when fibrinolysis begins may favour retraction that might otherwise have been prevented by adhesion

None of these rather vague speculations account for the most striking fact to emerge from nearly all the investigations of the retraction problem that the platelets are an essential component of whatever mechanism is concerned

THE ACTION OF THE PLATELETS

The evidence that the platelets are essential for proper retraction has already been reviewed and most workers have accepted it as conclusive though as might be expected denials can be found Howell (1916) for instance observed that pure fibrinogen clotted by thrombin produced clots which shrank away from the walls of the tubes But it is probable that the fibrinogen used in this experiment was sufficiently diluted to form wide meshed clots which simply collapsed so giving the impression of active retraction Budtz-Olsen (1951) has discussed this point and has shown that with a concentration of fibrinogen equivalent to that of normal plasma retraction does not occur unless platelets are added Ellicot and Conley (1951) have confirmed this

It is an old observation that during fibrin formation the platelets adhere to the fibrin and that small agglutinated masses form particularly at the intersection of the fibres Duke (1912) suggested that the action of the platelets in promoting retraction was the binding of one fibre to another Without platelets the contraction of *individual fibres (due presumably to some inherent property* of the fibrin itself) would not reduce the volume of the clot since the fibres would tend to slip one over the other The presence of platelets would cause by their adhesion the formation of firm knots at the intersection of fibres so that shortening of the fibrin would be effective in producing retraction This view is obviously held also by Tocantins (1936a) though in addition he describes a process of bending twisting and shortening which he associates in some way with platelet agglutination Consideration of this theory shows that it cannot provide an adequate explanation of the facts It is true that if there were no adhesion between the fibrin fibres any shortening they might undergo would have little effect on the volume of the clot But if there were in fact no adhesion between the fibres the clot itself would have no solidity and would disintegrate Budtz-

Olsen (1951) has shown that the clots formed in the absence of platelets though they do not retract, have as much mechanical strength as clots containing the normal number of platelets It seems, therefore, that the same amount of adhesion between fibrin fibres must be present in both types of clot Mere shortening of the fibrin fibres by a process similar to syneresis is an unlikely explanation of the extreme reduction in volume observed in plasma clots which is usually of the order of 95 per cent Even in a lattice in which all the intersections were knotted together so to speak, by platelets it would be necessary to postulate an average change in length of 60 per cent or more in individual fibres Such changes in length of fibrin fibres in the absence of platelets have not been observed under natural conditions

Glanzmann (1918) and Fonio (1951) favour the idea that there is a specific retraction factor (retractozyme) which acts on the fibrin, *in some way causing its shortening Fonio (1951) has claimed that* by ultrasonic disintegration he can break down platelets into a hyaline material which promotes retraction and a granular material which promotes coagulation It is suggested therefore that the retraction factor is derived from the hyaline material of the platelets On the other hand Rovatti (1950) found that ultrasonic treatment of plasma reduced subsequent retraction to a degree proportional to the amplitude of the vibration This reduction in retraction ran parallel with the degree of destruction of the platelets which was studied by electron microscopy

The Vital Activity of the Platelets

The remaining hypothesis relating the platelets to retraction suggests that platelet action is a vital one In 1873 Vulpian and Ranvier observed platelets changing in form during coagulation and sending out and pulling in long pseudopodia Bizzozero (188_) described how the platelets formed the knots in the fibrin network and noticed clear fluid escaping from them during their agglutination. Eberth and Schimmelbusch (1885) also noticed these morphological changes and believed that they indicated that the platelets were living cells Frank (1915) and Tait and Green (1926) believed that retraction was due to the shrinkage of masses of platelets adhering to the fibrin Bessis (1950) and Braunsteiner et al (1950) have published beautiful and convincing photographs obtained by electron-microscopy of the long pseudopodia extruded by platelets (see

Plate 2) From these observations and his own experiments Budtz Olsen (1951) has put forward a theory of clot retraction which depends on vital platelet activity He watched long filamentous pseudopodial processes being thrown out by the platelet masses formed during coagulation these processes being attached to the fibrin network and also linking up with processes sent out by other platelet masses He has observed in the absence of fibrin adjacent platelet masses to be drawn together apparently by the contraction of inter-connecting pseudopodia He believes that clot retraction is due not to any inherent property of the fibrin itself but to the contraction of filamentous pseudopodia thrown out by the platelets these pseudopodia being attached to the fibrin itself or to other pseudopodia from platelet agglutinates at the next fibrin inter-section He points out that in lower animals such as limulus coagulation of the blood is brought about not by fibrin but by a network of extrusions from special cells present in the plasma The mammalian platelet is analogous to this primitive thrombocyte and retraction may be a sort of vestigial function dating from a remotely primitive coagulation mechanism Added to these direct observations of the apparently vital activity of the platelets there is the indirect evidence that anything which might be expected to kill living cells will inhibit clot retraction Heating the blood to 45° C will render it non-retractile even if it is then cooled to the optimum temperature for retraction during coagulation Such procedures as storage freezing crushing exposure to short waves ultra-violet light or X-rays (Werner 1943 Arthus and Chapiro 1908) all inhibit retraction Destruction of the platelets by antiplatelet serum or sedormid in the case of sensitive patients also prevents retraction (Ackroyd 1949a) A number of substances such as opium (Thackrah 1819) chloroform cocaine methylene blue aniline blue methyl violet and trypan red which either stain or fix the platelets, prevent retraction though they do not necessarily prevent agglutination of the platelets (Archard and Aynaud 1908) Other evidence of the vital activity of the platelets is suggested by the fact that they like other living cells reduce methylene blue (Achard and Aynaud 1908) they consume oxygen (Endres and Kubowitz 1927) and glucose and release lactic acid (Tullis 1952) The observation by Fantl Ebbels and Nelson (1951) that substances that act on the thiol groups of platelets inhibit retraction could be interpreted as meaning that such groups are necessary for the vital action of the platelets

It seems likely therefore that a vital function of the platelets causing a mechanical shortening of fibrin fibres or of inter linking pseudopodia may account for clot retraction This platelet activity associated as it appears to be with morphological changes known as viscous metamorphosis is stimulated in some way by the process of coagulation Wright and Minot (1917) studied this metamorphosis and pointed out that it could be produced by fresh serum or by oxalated plasma after recalcification but not by a mixture of oxalated plasma and thrombin Budtz-Olsen (1951) has shown that mixtures of thrombin and fibrinogen or thrombin thromboplastin and fibrinogen do not produce the characteristic changes in the platelets nor retractile clots The addition of a prothrombin preparation and calcium to these mixtures promoted retraction and platelet metamorphosis It was concluded that some factor other than thrombin is produced during prothrombin conversion which potentiates platelet activity and retraction Bergsagel s (1956) observations on the platelet changes produced by the interaction of A H G Calcium and Christmas factor may be important from the point of view of retraction as well as that of thromboplastin generation However Ellicot and Conley (1951) have found that colloids such as human or bovine albumin and even gum acacia may have a similar retraction promoting effect on an otherwise non-retractile platelet-thrombin-fibrinogen mixture

The principal evidence against the vital theory of retraction is Fonio s (1951) claim that the hyaline sediment from platelets that have been disrupted ultrasonically promotes retraction Against this must be set the statement by Rovatti (1950) that ultrasonic treatment of the plasma inhibits retraction and destroys the platelets Perhaps Fonio s hyalomer fraction contained sufficient platelets that had escaped destruction to produce retraction

THE FUNCTION OF CLOT RETRACTION

It is usually supposed that clot retraction has some part in normal haemostasis mainly because in certain conditions in which retraction is impaired such as thrombocytopenic purpura and obstructive jaundice the patients may have manifest haemorrhagic tendencies It is also obvious that the retracted clot is denser and it is apparently tougher than the unretracted one Carr and Foote (1934) suggested that the deficient clot retraction was mainly responsible for the bleed

ing in jaundice Further consideration does not support this view In many cases of athrombocytopenic purpura there are severe haemorrhagic symptoms, very similar to those of thrombocytopenic purpura but clot retraction is normal (Macfarlane 1941) Deficient clot retraction in obstructive jaundice is related to an increased blood fibrinogen which sometimes occurs and is not closely related to the haemorrhagic tendency which is of course due to the effects of Vitamin K deficiency There are many instances of deficient clot retraction associated with increased fibrinogen concentration such as pneumonia, Hodgkin s disease myelomatosis pregnancy liver diseases and other conditions with no increased tendency to haemorrhage

Fonio (1921) believed that retraction acted as a 'physiological ligature of the vessels since during the process of shrinking a clot adherent to the walls of a vessel might be expected to draw the edges together Actually Budtz-Olsen (1951) has shown that the force exerted by retracting blood clots is extremely feeble so that even the slightest adhesion of the clot to the walls of a glass tube prevents retraction Kristenson (1932) suggested that shrinkage of the blood clot might be useful not in drawing the walls of a vessel together but by allowing a thrombosed vessel to become recanalized as a clot shrank away from part of its internal surface Quick (1950b) in a recent evaluation of the function of retraction comes to the conclusion that it has no part in haemostasis but points out that fibrin adsorbs thrombin very actively so that before retraction is complete no significant accumulation of thrombin occurs in the interstices of the clot Since thrombin itself favours thrombin generation the conversion of prothrombin in unretracted blood clots is slowed by thrombin adsorbtion When retraction begins thrombin begins to accumulate in the serum and rapid consumption of prothrombin then occurs From this it might be argued that retraction favours thrombin generation in the immediate vicinity of an intravascular thrombus and so promotes further thrombosis

SUMMARY TO CHAPTER VIII

Clot retraction, which follows coagulation has been measured by many different methods Retraction is influenced by the surface with which the blood is in contact, and by pH and temperature by

the packed volume of red cells in the blood by the number of platelets and by the concentration of fibrinogen

The mechanism of clot retraction is not well understood. It has been claimed that the process is similar to 'syneresis of colloidal gels but the speed and extent of retraction and the marked effect of temperature and the feeble force exerted by the clot during retraction, oppose this hypothesis Others have claimed that retraction is a stage in the digestion of the clot but fibrinolysis and retraction occur independently The view that live platelets are actively concerned in retraction is supported by the fact that procedures such as storage freezing crushing heating to 45 C exposure to short waves etc. which are likely to damage living tissues impede retraction Substances which fix platelets prevent retraction and the presence of antiplatelet serum inhibits retraction

The function of clot retraction is unknown

CHAPTER IX

FIBRINOLYSIS

INTRODUCTION

In the normal processes of haemostasis and repair of wounds blood clots are not permanent structures Coagulated blood is a temporary haemostatic barrier and possibly a supporting framework for the growth of fibroblasts and new capillaries but as the new tissue forms the fibrin clot disappears Intravascular thrombi may also disappear quite rapidly so the thrombosed vessels become recanalized The body possesses therefore the means for disposing of unwanted fibrin This may be partly achieved by proteolytic enzymes derived from the tissues and the leucocytes but there is now evidence that the blood itself contains one or more precursors of proteolytic and fibrinolytic factors which are potentially of great power One of these enzymes (plasmin) is present in such concentration that when fully activated it would be capable of digesting the total fibrinogen of the body in a few minutes

It is now recognized that fibrinolytic activity can be induced in the blood by a number of quite different processes Normal plasma for instance when treated in vitro with certain bacterial filtrates or with chloroform develops a powerful fibrinolytic and proteolytic activity The blood of human beings and animals taken post mortem, particularly in cases of sudden death can often be shown to be capable of liquifying fibrin and there are many observations which suggest that any form of stress mental as well as physical can induce fibrinolytic activity in the blood of living human subjects The phenomenon of fibrinolysis can therefore be the result of diverse causes and it is not yet clear whether or not the actual process of fibrin destruction is always due to the action of the same lytic factor Since the subject may have ultimate physiological and pathological importance it is considered here but it is emphasized that only a brief summary of the situation can be given as bacteriological and biochemical ramifications cannot adequately be dealt with A review of the literature to 1947 was published by Macfarlane and Biggs (1948) and recent work has been described by Astrup (1956a)

FIBRINOLYSIS PRODUCED IN VITRO

The effect of Chloroform

In 1889 Denys and Marbaix found that a proteolytic activity developed in serum after the addition of chloroform ether or thymol a phenomenon investigated in greater detail by Delezenne and Pozerski (1903) It was found that serum treated in this way digested gelatin and casein its action being inhibited by the addition of untreated serum It was later found by Dale and Walpole (1916) that chloroform destroys the antiproteolytic power of normal serum and it seemed possible that the removal of an inhibitor by chloroform revealed the presence of a proteolytic factor normally present in the blood Further study by Tagnon (1942) and Tagnon Davidson and Taylor (1942) demonstrated that this proteolytic factor is associated with the globulin fraction of the plasma is actively fibrinolytic and is capable of destroying fibrinogen

Fractionation

Green (1887) found that separated fibrin prepared from ox blood lysed spontaneously when suspended in saline He showed that this was an irreversible process as the dissolved fibrin could not be clotted again by thrombin Hedin (1904) and Opie and Barker (1907) found that the globulin fraction of the plasma prepared by salt precipitation might show spontaneous proteolytic activity which was inhibited by the albumin fraction Feissly (1942) and Macfarlane and Pilling (1946b) have made similar observations and Taylor et al (1945) have found that plasma fractions prepared by the Cohn process may have fibrinolytic activity Schmitz has prepared a fibrinolytic enzyme from plasma by precipitation with trichloroacetic acid It appears therefore that either destruction of an inhibitor by chloroform or its removal by fractionation may reveal a latent fibrinolytic and proteolytic activity in normal blood

Activation by Bacterial Filtrates

In 1933 Tillett and Garner found that if human plasma clots in the presence of certain strains of beta-haemolytic streptococci very rapid fibrinolysis occurs Milstone (1941a) showed that fibrin derived from pure fibrinogen was resistant to the action of strepto-coccal culture filtrates but that if a small amount of the globulin fraction of human plasma were added rapid lysis occurred He postulated a lytic factor in the globulin fraction required by the

filtrate for its action Kaplan (1944) and Christensen (1945) and Christensen and MacLeod (1945) then produced evidence suggesting that lytic factor contained the precursor of a proteolytic enzyme that was activated after the addition of the bacterial filtrate The name streptokinase was coined for the bacterial factor 'plasmin for the proteolytic enzyme and plasminogen for its precursor A natural inhibitor of fibrinolysis present in the albumin fraction of the plasma became antiplasmin Lewis and Ferguson (1951) have shown that fibrinolytic activity can be produced presumably by a similar mechanism by the action of 'staphylokinase on the blood of dogs

Though this work provided an important advance in the recognition of a proteolytic enzyme system existing in normal human blood attempts to study the quantitative relationships of the supposed streptokinase – plasminogen – plasmin system led to anomalous results For instance using gelatin as a substrate Christensen and MacLeod (1945) concluded that the activation of human plasminogen to plasmin followed a first order reaction the rate of change being proportional to the concentration of streptokinase which apparently acted as a catalyst Ratnoff (1948) and Wassermann (1952) who used bovine fibrinogen as a substrate considered however that streptokinase reacts stoichiometrically with human plasminogen There were also inexplicable species differences Though human plasminogen preparations readily developed proteolytic activity on the addition of streptokinase bovine plasminogen could be activated by it only slightly or not at all If however a mixture of human plasma globulin and streptokinase were added to bovine plasminogen active plasmin was formed which was derived from the bovine plasminogen not from the human globulin (Müllertz and Lassen 1953)

These discrepancies have been explained by Müllertz (1955) and Müllertz and Lassen (1953) who have produced evidence that the activating system is more complex than originally supposed a situation all too familiar to the worker in blood coagulation research It seems likely that streptokinase does not act directly upon plasminogen but upon an inert proactivator which is thus transformed to 'activator The activator then reacts with plasminogen to form plasmin

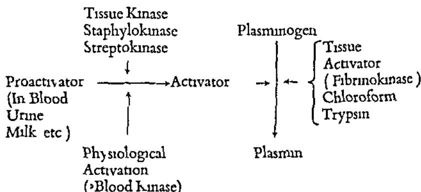
The previous failure to recognize this additional stage had led to the difficulties mentioned. Streptokinase does not activate bovine

plasminogen because there is very little proactivator in this material most human plasminogen preparations on the other hand, contain large amounts of pro-activator and will thus not only form plasmin on the addition of streptokinase but will also develop the ability to activate bovine plasminogen Mullertz (1955) concludes from his observations that formation of activator by the reaction between streptokinase and proactivator is stoichiometric and the transformation of plasminogen to plasmin is catalysed by the activator Thus the total process of the production of plasmin under the influence of streptokinase might appear to be either stoichiometric or catalytic if one or other of the two reactions involved was the limiting factor In past work in which the proactivator-activator system was unsuspected the results obtained were confusing or apparently conflicting since proactivator might or might not be present in the plasminogen preparations or in the fibrinogen substrate often used as an indicator but not in substrates such as casein or gelatin.

Activation by Tissue Extracts

There is a voluminous literature on the presence of proteolytic and fibrinolytic enzymes in tissue extracts (Hedin 1904 Abderhalden 19-1 Rosemann 1936 etc.) but it is Astrup and his colleagues who have shown that such tissue extracts probably owe their effect to their ability to activate a proteolytic system in the blood (Astrup and Darling 1943 Astrup and Permin 1947 1948 Astrup 1948 1951 1956 Astrup Crockston and MacIntyre 1950) Many different tissues contain this activator, pig s heart being the best source and among human tissues lung and brain having the greatest activity Fibrinolytic activity was also ascribed to various secretions Macfarlane and Pilling (1950) described a fibrinolytic activity in urine which was shown by Williams (1951) to be mainly due not to a fibrinolytic enzyme, but to the presence of an activator which was capable of activating the plasminogen contained in most fibrin substrates used to demonstrate fibrinolysis The existence of this activator in urine was confirmed by Astrup and Sterndorff (1952) and it was also found in human milk (Astrup and Sterndorff 1953) tears (Storm 1955) and saliva (Albrechtsen and Hess Thaysen 1955) In these latter fluids the activator was also present in an inert precursor form which could be activated by streptokinase This observation at once links these activator systems with that of the blood, which responds in a similar way to the action of streptokinase

In the case of tissue extracts the action on plasminogen is direct and Astrup and his co-workers at first used the term 'fibrinokinase' to describe this form of plasminogen activator but now refer to it as 'tissue activator'. It is not clear if 'fibrinokinase' and the activator generated in blood or body fluids are one and the same agent, but in rather different physical states. Though Astrup and Stage (1952) have described a soluble preparation, fibrinokinase activity is mostly retained in the insoluble tissue proteins of the so-called microsome fraction (Astrup and Permin 1947), whereas the activator of the blood and body fluids is in solution. Even if these activating agents differ, however, Mullertz (1955) has produced evidence that they activate the same pro-enzyme plasminogen and previous indications that different proteolytic system existed in the blood were due to the fact that tissue activator (fibrinokinase) and the activator of the blood react directly with plasminogen, whereas streptokinase and certain other agents known collectively as lysokinases only convert proactivator to activator, as shown in the scheme below.



POST MORTEM FIBRINOLYSIS

The incoagulability of the blood in cases of sudden death has been recognized since the writings of Morgagni (1769) and Hunter (1794). Morawitz (1906) seems to have been the first to demonstrate that such blood lacks fibrinogen and may contain an active fibrinolysin. In dogs killed by asphyxia similar changes could be observed (Fidon, Gautier and Martin 1908). Judin (1936) describes the practical application of this phenomenon in the organization of a Russian corpse-blood transfusion service, in which the natural lysis of the blood from suitable donors made anticoagulants unnecessary.

Mole (1948) provided the first systematic examination of this phenomenon He found an active fibrinolysin in the blood of 90 per cent of post mortem cases in which the blood was fluid after death This lysin could be separated by precipitation by ammonium sulphate or trichloroacetic acid. Halse (1948) studied post mortem fibrinolysis in cats and rabbits killed by asphyxia or drowning and observed that the longer the blood circulation continued after cessation of respiration the greater the lytic effect He also observed an increase in parallel with the fibrinolysin activity of blood phosphatide and blood sugar Berg (1950) investigated fibrinolytic activity in 143 human post mortem cases and found it most marked after death from asphyxia and haemorrhage He confirmed that in such cases there was an increase in the blood phosphatide and also of adrenalin-like substances

FIBRINOLYSIS IN VIVO

The main obstruction to the study of fibrinolytic activity produced experimentally in animals is the extreme severity of the procedures required. The fibrinolytic effect therefore is complicated by a number of others which makes analysis difficult. Dastre (1893) who seems to have been the first to use the term fibrinolysis observed the disappearance of clots from the blood taken from dogs subjected to severe haemorrhage Tagnon et al (1946) observed fibrinolysis in human cases of haemorrhage and severe burns and following haemorrhage in dogs Nolf (1908 1938) and Nolf and Adant (1950) have made observations on the extreme fibrinolytic activity produced by the injection of peptone into dogs following the removal of the liver or its isolation from the circulation In these experiments many other factors are clearly operating Fibrinolysis in animals has also been produced by means of anaphylactic shock (Imperati 1939 Rocha e Silva et al 1946) tourniquet shock (Westphal et al 1950) and electrically induced convulsions (Fantl and Simon 1948)

Macfarlane (1937) observed increased fibrinolytic activity in human patients after surgical operation, using a dilution technique which has proved to be a sensitive indicator of such changes These observations were confirmed and extended by Imperati (1937 1939) von Kaulla (1947) and Halse (1948) Macfarlane and Biggs (1946) found that the fibrinolytic activity was more related to the mental distress of the patient than to surgical trauma in operation cases and

following this up, Biggs, Macfarlane and Pilling (1947) showed that severe exercise or the injection of adrenalin, had similar effects It has also been observed during air raids and in anxiety states (Latner 1947) in students about to take part in examinations and even in a patient receiving alarming suggestions under hypnosis (Truelove 1951)

Kwaan and McFadzean (1956) have observed that fibrinolytic activity develops in the blood in the human arm distal to a tourniquet causing complete occlusion of the circulation and consider that ischaemia produces local changes causing fibrinolytic activation, since no fibrinolysis was observed in blood taken at the same time from the other (non-occluded) arm One source of possible confusion has been revealed by Fearnley Revill and Tweed (1952) and Truelove (1953) who have shown that fibrinolytic activity arising in the blood in vivo is extremely labile and that unless tests are set up immediately after obtaining the blood, or special precautions are taken to preserve activity such as immediate refrigeration falsely negative results will be obtained

CORRELATION OF OBSERVATIONS ON FIBRINOLYSIS

The work of Kaplan (1944) Christensen (1945) and Christensen and MacLeod (1945) seems to have established that the fibrinolytic activity generated in blood by the action of chloroform and by streptokinase is due to the appearance of the same proteolytic enzyme plasmin' This enzyme is derived from its precursor, plasminogen which is present in the globulin fraction of the plasma, and contaminates most fibrinogen preparations unless special precautions are taken to remove it (Kekwick, Mackay and Nance 1955) Plasmin is capable of digesting fibrin fibrinogen casein and gelatin has a pH of maximum activity and stability of 7 2 and its action is inhibited by soya-bean and pancreatic trypsin inhibitors and by a natural inhibitor present in the albumin fractions of the plasma It is generally supposed that chloroform promotes the activation of plasminogen by some intrinsic activator system by destroying the inhibitor which normally keeps this system in check. Streptokinase converts the proactivator of the plasma to activator which then catalyses the conversion of plasminogen to plasmin Fibrinokinase usually prepared from tissues activates plasminogen directly Since human fibrinogen is usually contaminated with both proactivator and plasminogen, fibrin formed from it will undergo lysis in the presence

of either streptokinase or fibrinokinase bovine fibrinogen however lacks proactivator but contains plasminogen and will thus form a lytic system with fibrinokinase but not with streptokinase

Evidence has been produced by Bidwell (1953) suggesting that the fibrinolytic activity which develops spontaneously in vivo following exercise or stress is due to an agent similar to that studied by Mole (1948) in post mortem blood Bidwell and Macfarlane (1951) had already suggested that this activity might be due to the appearance of a 'kinase which activated plasminogen during the clotting process rather than to the direct action of a proteolytic enzyme activated in vivo Bidwell (1953) and Mole (1948) both observed that the fibrinolytic activity developing sponstaneously in man differed from that of plasmin since the former appeared to have no effect on fibrinogen or such substrates as casein and gelatin and similar differences were found by Fantle and Simon (1948) in observations on fibrinolysis induced in animals by electric convulsions

It seems likely however that these difficulties and the apparent dissimilarity between the phenomena of spontaneous fibrinolysis post mortem fibrinolysis and plasmin activity can be explained by the unsuspected complexity of the plasmin activating system Mullertz (1953) has found increased amounts of activator in blood undergoing spontaneous fibrinolysis thus confirming the suppositions of Bidwell and Macfarlane (1951) that the activity was due to the presence of a kinase

Such an activator would not of course cause digestion of substrates such as casein or gelatin which do not contain plasminogen its inability to digest fibrinogen is probably explained by its rapid inactivation by an inhibitor while in solution in plasma while its selective adsorbtion with plasminogen to fibrin during the process of clotting would protect it from the action of the inhibitor and promote subsequent fibrinolysis

THE MECHANISM OF ACTIVATION IN VIVO

It is probable that fibrinolytic activity developing spontaneously in the blood of the living subject is due to the release into the blood stream of lysokinases which like streptokinase are capable of producing plasminogen-activators from the proactivator of the blood or of fibrinokinase like that extracted from tissues which are capable of acting directly on plasminogen Certainly in the defibrination

syndrome due to the entry into the blood stream of tissue fragments derived from the placental site or from damaged lung tissue intense fibrinolysis is often observed Halse (1948) noted the increased concentration of phosphatides in fibrinolytic blood from asphyxiated animals and suggested that a phosphatide might be the activator since he had observed activation of the fibrinolytic system by thromboplastic (phosphatide-containing) substances

There is also evidence that a similar mechanism may be concerned with the activation in vivo which is produced by mental or physical stress or the injection of adrenalin since Berg (1950) found that there was an increase of blood phosphatide and active fibrinolysis in human subjects undergoing electric convulsion therapy and following the injection of adrenalin Nevertheless the action of adrenalin in activating the fibrinolytic system is not clear since fibrinolysis and adrenalin activity are not interdependent Truelove (1951) found that though adrenalin in sufficient amounts causes both fibrinolysis and eosinopenia smaller doses will cause eosinopenia without fibrinolysis whereas exercise or mental stress may cause intense fibrinolysis but no eosinopenia and thus it might be argued little rise in blood adrenalin It is probable therefore that adrenalin is not an essential link in the mechanism which activates the fibrinolytic system The observation of Kwaan and McFadzean (1956) that fibrinolytic activity can develop locally in occluded vessels is of great interest It suggests that the activator may be derived from the peripheral tissues or even from vascular endothelium as a result of ischaemia and should provide a new approach to the problem of fibrinolysis in vivo

THE MEASUREMENT OF FIBRINOLYSIS

Early observations on fibrinolysis were made with whole blood or recalcified citrated or oxalated plasma In some cases as after sudden death occasionally during life and in certain animal experiments the activity is sufficient to destroy the natural blood clot within an hour or two Macfarlane (1937) used a dilution technique which was more sensitive in demonstrating smaller increases in fibrinolytic activity If was found that normal plasma diluted 1 in 16 or 1 in 32 with saline produced fine webs of fibrin that remain undissolved for many days but in the stress conditions already mentioned, lysis took place in a few minutes to several hours Subsequent clinical and experimental observations by Imperati (1935) Macfarlane and Pilling

(1946b) Biggs Macfarlane and Pilling (1947) Mole (1948) Truelove (1951) and Williams (1951) have been made by this method The lability of the activity demonstrated by Truelove (1953) and Fearnley Revill and Tweed (1952) has emphasized the necessity for refrigeration of the blood and plasma until the test is actually set up The method which depends on the fact that dilution favours fibrinolysis possibly by depressing inhibitory factors gives only roughly quantitative results and indicates over-all changes in the fibrinolytic activity of the blood, and does not point to any one of the factors which may be involved.

More quantitative results can be obtained by measuring in various ways the amount of fibrin lysed in a given time Halse (1948) estimated the amount of fibrin remaining and the amount of protein that had dissolved by nitrogen determinations on the residual clot and the supernatant fluid after a period of lysis He has also developed a colorimetric method using fibrin dyed with carmine red, and estimating the dye released by lysis Bidwell and Macfarlane (1951) have used a quantitative method for assay of natural fibrinolytic activity based on measurement of the amount of fibrin remaining after a given period of lysis (See appendix) Other methods depending on the lysis of fibrin have been used. In one the tube method the time required for the lysis of a fibrin clot in a test tube is taken as the index of activity (Rocha e Silva and Rimington 1948) Astrup and his colleagues have used plates of fibrin on which drops of the test preparations are placed. Lysis produces a spreading zone of liquefaction and the area of this related to time gives a quantitative measurement This test can be made to indicate the nature of the lytic agent Plates made from bovine fibrin in which plasminogen has been destroyed by heat will only be lysed by agents such as plasmin which are directly fibrinolytic or proteolytic plates made from unheated bovine fibrin (standard plates') contain plasminogen and thus are lysed by plasminogen-activators such as fibrinokinase as well as by plasmin and plates made from human fibrin contain both plasminogen and pro-activator and are thus lysed by lysokinases such as streptokinase as well as by fibrinokinase and plasmin (Permin 1947 1949 1950b Astrup Crockson and MacIntyre 1950 Müllertz 1955 Astrup 1956)

In most of the work on fibrinolysis induced by streptokinase or chloroform substrates other than fibrin have been used to demonstrate a general proteolytic activity Christensen (1945) has used

viscometric determinations to measure digestion of all these substrates and also estimations of the increase in acid soluble tyrosine and amino-nitrogen Troll Sherry and Wachman (1954) have used amino-acid substrates to study the action of plasmin It has not been practical to use these methods in the investigation of physiological fibrinolysis probably because fibrin by its strong adsorbtion of fibrinolytic factors is a much more susceptible substrate and thus reacts when other substrates remain unchanged

THE SIGNIFICANCE OF FIBRINOLYSIS

Only conjectures are available on the possible significance of fibrinolytic activity occurring spontaneously in human subjects An obvious possibility is that it is concerned with the removal of fibrin clots in wounds or inflamed tissues when their term of usefulness is over The finding of fibrinolytic systems in milk urine tears and saliva as well as in the blood suggests that the maintenance or restoration of the patency of vessels and ducts in general may depend on the removal of fibrin which may block them following trauma or infection

There have been suggestions that a proteolytic enzyme is concerned in blood coagulation Nolf (1908 1938) believed that proteolysis is a factor in normal blood coagulation It has been known for many years that trypsin will clot oxalated blood and Ferguson and Erikson (1939) have shown that trypsin causes an increased rate of conversion of prothrombin to thrombin Lenggenhager (1946) believes that a proteolytic factor is concerned in the initial stages of prothrombin conversion Feissly (1942) obtained a proteolytic component from plasma which had the power to convert prothrombin to thrombin in the presence of calcium It has also been observed that inhibitors of trypsin delay coagulation Ferguson (1942) showed that pancreatic trypsin inhibitor prevents coagulation and Macfarlane and Pilling (1946a) and Macfarlane (1947) showed that soya-bean trypsin inhibitor had an anticoagulant action which was apparently due to inhibition of thromboplastic activity Thus proteolysis favours coagulation but it has not been possible to show directly that a proteolytic factor is a normal component of the clotting mechanism as now recognized It has been positively shown (Ratnoff Hartmann and Conley 1950) that a globulin fraction of plasma with proteolytic activity can be prepared which has no prothrombin thrombin fibrinogen thromboplastin or Factor V activity We

ourselves have not been able to demonstrate any accelerating action of plasmin preparations on thrombin generation in either whole blood or any mixtures of isolated coagulation factors but it must be recognized that the mechanism of intrinsic thromboplastin generation has not yet been studied in detail and may involve hitherto unknown factors

Rocha e Silva et al (1946a b c) have suggested that fibrinolysis may take part in the development of anaphylaxis They have shown that trypsin perfused through an isolated liver causes the release of histamine though perfusion with an anaphylactic antigen caused no release of histamine from the livers of sensitized animals unless it was perfused together with whole blood Since anaphylactic shock is associated both with a rise in blood histamine and fibrinolysis it was suggested that the antigen released fibrinolysin which in turn released histamine. Ungar (1947) showed that incubation of sensitized tissue with the specific antigen does cause the release of a proteolytic enzyme

The observation that increased fibrinolysis occurs in anxiety states and in conditions associated with prolonged mental or physical stress suggests that there may be some association between the increase of proteolytic activity and the disturbed metabolism in these conditions In such cases there may be a greatly increased breakdown of body protein causing loss of weight and an adverse nitrogen balance It is tempting to suggest that the fibrinolytic activity demonstrable in vitro reflects an increased proteolytic tendency of the circulating blood which disturbs the equilibrium of protein metabolism.

It is possible that fibrinolysis may be important in patients with thrombosis and embolism The increased fibrinolytic activity now known to be associated with bodily trauma or anticipated trauma may have some function in limiting the extent of coagulation in damaged vessels and in keeping undamaged vessels free from deposits of unwanted fibrin Or fibrinolytic activity by loosening clots already formed may perhaps predispose to embolism. Only careful studies of the time relationship of changes in fibrinolytic activity and the onset of thrombotic or embolic episodes can determine these possibilities

Finally there have been suggestions that increased fibrinolysis may be a cause of abnormal haemorrhage Reimann (1941) described a haemorrhagic purpura in a woman of fifty-eight associated with

increased fibrinolysis He called this condition 'thrombolytic purpura' There have also been described a number of cases of an acute haemorrhagic state occurring in pregnancy and associated with placental detachment or foetal death and following lobectomy (see Chapter XIII) In such cases defibrination of the blood and active fibrinolysis has been described (Schneider 1951 Soulier et al 1952 Favre-Gilly 1952) It is theoretically possible that an intense fibrinolytic activity could deplete the fibrinogen of the blood though an alternative explanation would be that the absorbtion of thromboplastic placental or lung tissue may have caused the diffuse intravascular coagulation which has been described in the so-called 'negative-phase reaction (see Chapter XIII)

Tagnon et al (1952) have studied the intense fibrinolytic activity that may occur in certain cases of carcinoma of the prostrate with metastases and which may explain the haemorrhagic tendency which occurs in such patients

SUMMARY TO CHAPTER IX

A proteolytic enzyme system can be found in human plasma Active enzyme can be prepared from the globulin fraction and an inhibitor from the albumin The mechanism for the activation of this system is complex and much confusion has arisen from the use of impure substrates The active enzyme (plasmin) will act on a variety of substrates such as fibrin fibrinogen casein and gelatin The enzyme is derived from a precursor (plasminogen) the conversion of plasminogen to plasmin is brought about by various complicated activation systems. Tissue extracts (said to contain fibrinokinase or tissue activator) convert plasminogen to plasmin directly Substrates which contain plasminogen (such as human or bovine fibrin) will be lysed by tissue extracts but tissue extracts will not act on casein or gelatin In the past these extracts have been said to contain a powerful fibrinolytic enzyme highly specific for a fibrin substrate but they are themselves only feebly proteolytic they activate the plasminogen present in fibrin Various secretions such as urine tears milk and saliva also contain activators for plasminogen the activating power of these secretions is greatly increased by various bacterial filtrates (streptokinase staphylokinase) It is con-

cluded that the plasminogen activator in these secretions is present partly in an inactive form (proactivator) which is activated by bacterial filtrates. Substrates which contain proactivator (such as human fibrin) will be lysed by streptokinase because the proactivator is converted to activator and this in turn activates plasminogen and the plasmin lyses the fibrin. Substrates which contain little proactivator (such as bovine fibrin) will not be lysed by streptokinase. Bovine fibrin may be freed from plasminogen and proactivator by heating. Such heated fibrin is lysed by plasmin but not by tissue extracts or by streptokinase.

Fibrinolysis occurs during life and this activity may be due to the liberation into the blood of activators of the fibrinolytic system. Activity is detected after electroconvulsive therapy, after adrenalin injections, following mental stress, and in the acute defibrination syndrome. Fibrinolytic activity can be measured by recording the amount of fibrin lysed in a given time. There are many conjectures about the physiological significance of the fibrinolytic system.

CHAPTER X

ARTIFICIAL ANTICOAGULANTS AND DECALCIFYING AGENTS

To study the coagulation of blood and to obtain samples of blood for biochemical and other tests it is often necessary to prevent coagulation by the addition of an artificial anticoagulant A number of anticoagulants have been used for this purpose In addition anticoagulant drugs have been used to prevent coagulation in the blood of patients with thrombosis In this chapter it is proposed to discuss the anticoagulants which have been used in vitro In Chapter XVIII the clinical use of anticoagulant drugs will be described

THE ANTICOAGULANT ACTION OF NEUTRAL SALTS

Coagulation can be prevented by the addition of a sufficient amount of neutral salts to the blood immediately it is withdrawn from the body Most salts have some anticoagulant effect and use is made of this fact in the collection of fluid blood samples for pathological tests

Arthus and Pagès (1890) and Pekelharing (1892) showed that *oxalate and citrate solutions were very effective anticoagulants* These two ions form unionized calcium salts and the anticoagulant action of the anions can be neutralized by the addition of calcium From this work it was generally assumed that the anticoagulant action of oxalate and citrate ions was due to the removal of calcium and that calcium was an essential factor in blood coagulation Since blood to which citrate or oxalate has been added will clot readily in the presence of thrombin and since thrombin and fibrinogen freed from calcium will react together it was further assumed that calcium ions facilitate some reaction leading to thrombin formation

These conclusions have never been doubted but several observations have suggested that the neutralization of calcium may not be the only effect of neutral salts on blood coagulation. Though oxalate and citrate ions removed calcium ions the amount of oxalate or citrate that must be added to blood to prevent coagulation is

much larger than the amount necessary to react with the free calcium ions present (Vines 1921) Moreover, anions which form relatively soluble calcium salts are also anticoagulant if used in sufficient concentration Lovelock and Porterfield (1952) have studied the effect of anions on blood coagulation from a new point of view They have found that there are two distinct anticoagulant effects of anions Using the anions which form calcium salts of relatively high solubility they found that the anticoagulant action was proportional to the valency Trivalent ions were more anticoagulant than divalent or univalent ions Secondly anions which form calcium salts of low solubility have a specific anticoagulant action These authors reduced the ionic strength of plasma by dialysis against distilled water and then increased the concentration of various ions They found that plasma would not clot if the ionic strength was reduced below 0 01 millimolar As the ionic strength increased to 0 03 millimolar an optimum was reached when a minimum amount of calcium would induce coagulation These authors have found that when various concentrations of $CaCl_2$ are added to saline dilutions of plasma the clotting time of the mixtures is proportional to the concentration of $CaCl_2$ and not to the concentration of plasma. If calcium entered into a quantitative union with a plasma constituent the clotting time should vary with the concentration of plasma They therefore conclude that calcium is not quantitatively involved in blood clotting as has been suggested by Quick (1947e and f)

Lovelock and Porterfield (1952) have found that coagulation occurs most readily when the proportion of calcium ions is $\frac{1}{16}$ of the total ionic strength Thus increase in ionic strength or decrease in calcium will both increase the coagulation time Thus citrate and oxalate ions which achieve both effects will be particularly powerful anticoagulants They conclude from their work that calcium acts in an adsorbed state and that the adsorbed calcium maintains a surface charge on one or other of the plasma colloids which is optimum for its proper interaction with other plasma colloids The work of Lovelock and Porterfield (1952) is a little difficult to reconcile with that of Bergsagel (1955) and Bergsagel and Hougie (1956) The latter authors think that calcium may react quantitatively with the Christmas factor However the two groups of workers approached the problem from very different angles and to settle this question new experiments in which both sets of observations are taken into account are required

In addition to the effect on thrombin formation neutral salts inhibit the thrombin-fibrinogen reaction if the concentration is sufficiently increased

NEUTRAL SALTS AND THE COLLECTION OF BLOOD SAMPLES

For the collection of blood specimens the salts most commonly used are sodium potassium, or ammonium oxalate sodium citrate or sodium fluoride The oxalate and citrate ions are usually used in blood coagulation work For most coagulation experiments citrate is to be preferred to oxalate both because the neutralization of calcium by citrate ions is more rapid than by oxalate ions and because Factor V does not deteriorate so rapidly in citrated as in oxalated plasma

Neutral salts have disadvantages for the collection of haematological samples because of their osmotic effect An ingenious combination of ammonium and potassium oxalate devised by Heller and Paul (1934) avoids this difficulty The ammonium ion causes the cells to swell and the potassium ion counteracts this effect. The proportion of the two salts used is 6 to 4 (Wintrobe 1951)

Sodium citrate is used for the collection of blood for transfusion A mixture found in practice to be useful is

Disodium citrate	2 g
Dextrose	3 g
Water	30 ml

to be mixed with 510 ml of blood (Mollison 1951)

Sodium fluoride is used for the collection of blood samples for the measurement of blood sugar The fluoride poisons the cell enzymes and prevents the metabolism of sugar during storage prior to carrying out the test Plasma rendered incoagulable with fluoride will not clot on recalcification because calcium fluoride adsorbs prothrombin and Factor VII

DECALCIFICATION WITH ION EXCHANGE RESINS

Quick (1947f) found that if blood was passed through the resin amberlite IR 100 which had been put into the sodium cycle then calcium was removed from the blood and coagulation was pre-

vented. This method of obtaining blood for the study of coagulation has the great advantage that no foreign substance is added to the blood It is true that cations other than calcium may be removed from the blood but this method of decalcification certainly produces less change in the blood than the addition of citrate which not only neutralizes the effect of calcium but also damages the white cells probably by interfering with enzyme action The ion exchange resins are now being used on a large scale in America for the preparation of blood and plasma fractions for transfusion (University Laboratory of Physical Chemistry Boston Red Book, 1950)

MISCELLANEOUS ANTICOAGULANTS

HEPARIN

Heparin is an anticoagulant because it interferes with the thrombin-fibrinogen reaction and with the formation of blood thromboplastin it is therefore not used in work on blood coagulation Heparin has no osmotic effect and is therefore an ideal anticoagulant for such tests as the measurement of packed cell volume or red cell fragility The mode of action of heparin is discussed in Chapter VII

SEQUESTRINE (Ethylene diamine tetra acetic acid disodium salt)

This substance is an anticoagulant by virtue of its calcium binding capacity It is specially useful for the study of platelets (Proesche 1951)

LIQUOID (Hoffman – La Roche)

Liquoid is an anticoagulant synthesized by Demole and Reinert (1930) In vivo this drug is toxic but in vitro it is often used for the collection of specimens for blood culture (Stuart 1948) Liquoid destroys complement and prevents the phagocytosis of bacteria by cells The specimen can be used for both culture and agglutination tests

ANTICOAGULANT DYES

Azo dyes such as Chicago sky blue and Chlorazol fast pink are anticoagulant (Huggett and Silman 1932 Huggett and Rowe 1933) Huggett and Silman (1932) made experiments which suggest that Chicago sky blue acts by neutralizing thromboplastin

Though Cholorazol fast pink is non toxic in that Huggett and Rowe (1933) showed that 16 mg /kilo had an anticoagulant effect whereas the M L D was 300 mg /kilo these dyes have not been much used Quick (1942) points out that the anticoagulant dyes contain sulphuric acid ester groups and suggests that some of the anticoagulant effect may be due to a heparin-like action

SOYA-BEAN AND PANCREATIC TRYPSIN-INHIBITORS

The anticoagulant action of pancreatic trypsin-inhibitor was studied by Ferguson (1942) and Grob (1943) Grob suggested that the anticoagulant action might depend on an inhibition of thrombin formation In 1944, Ham and Sandstedt and also Bowman found that soya-bean contained an anti-trypsin and Macfarlane and Pilling (1946a) and Macfarlane (1947) studied the anticoagulant action of a purified soya-bean inhibitor prepared by Kunitz (1945) and came to the conclusion that the substance was anti-thromboplastic, a conclusion which is supported by the observations of Biggs Douglas and Macfarlane (1953) who showed that the inhibitor destroys formed blood thromboplastin Glendenning and Page (1951) reinvestigated the anticoagulant action of soya-bean trypsin inhibitor and thought that the inhibitor formed a dissociable complex with prothrombin In mixtures the yield of thrombin was apparently related to the inhibitor level The investigation of these inhibitors is of theoretical interest but as yet no practical use has been made of them

COBRA VENOM

The venoms of most snakes are coagulants by their thrombin- or thromboplastin-like actions The venom of the cobra is an exception in that it is an anticoagulant which apparently delays thrombin formation (Mellanby 1909) by interfering with the action of brain thromboplastin (Kruse and Dam 1950) and of platelets (O Brien 1956)

HIRUDIN

In 1883, Haycraft observed that hirudin which occurs in the cervical glands of the leech was an anticoagulant Its mode of action has not been studied in recent years Mellanby (1909) thought that hirudin was both an antithrombin and an antithromboplastin

SUMMARY TO CHAPTER X

(1) Neutral salts are used for the collection of blood samples for investigation These salts prevent coagulation probably by interfering with the normal balance between calcium and other ions The optimum conditions for coagulation occur when the proportion of calcium ions is $\frac{1}{16}$ of the total ionic strength Neutral salts act both by increasing the total ionic strength and in some instances by a specific reduction in calcium ions

(2) Salts containing citrate and oxalate ions are most widely used for the collection of blood samples

(3) Decalcification of plasma with ion exchange resins may become an important method for the collection of fluid blood for transfusion

(4) Heparin and liquoid have a limited use for the collection of blood samples when changes in red cell size are to be avoided or for blood cultures

(5) Some miscellaneous anticoagulants of no practical importance are mentioned briefly

CHAPTER XI

BLOOD COAGULATION THEORY AND TESTS OF CLOTTING FUNCTION

The second half of this book attempts to apply the more theoretical knowledge about blood coagulation to the diagnosis and treatment of patients There is little initial difficulty in classifying patients into a few well recognized categories because much of the theory is based on the study of these patients and methods for the detection of their defects follow directly from the theory The techniques discussed in this chapter are designed for the study of these well established varieties of clotting defect When a patient is encountered whose abnormality does not coincide with that of any recognized defect serious confusion may arise unless the whole basis of blood coagulation theory is correctly appreciated. New factors will be proposed on inadequate evidence and the confusion multiplied by attempts to test for factors whose existence is doubtful The essential feature of blood coagulation theory is its cumulative nature the experiments of one group of workers is added to those of others and gradually a pattern of consistent results emerges and the probability of some underlying causal factor increases

THE EVOLUTION OF A CLOTTING FACTOR

If all else is confusion it is clear that blood coagulation involves a chain of reactions the final stages of which are reasonably defined Coagulation is ultimately caused by the polymerization of fibrinogen molecules brought about by an enzyme thrombin which is itself derived from a precursor prothrombin A very great deal of work has been done on these reactions during the last fifty years It is reasonably certain that the coagulation factors prothrombin and fibrinogen and their derivatives thrombin and fibrin involved in these reactions are true and real entities and that the reactions that they undergo are truly appreciated. All reactions which precede the conversion of prothrombin to thrombin are viewed hazily through a fog of intermediate stages because they must all be judged by their effect on clotting Moreover none of these early reactions

have been deduced wholly from experiments on normal blood Investigation has been initiated in every instance by the study of pathological blood and the factor or factors defined negatively as a substance deficient in the blood of a certain class of patient Subsequent work has incorporated many of these factors into the normal physiological process by creating artificial systems lacking the particular factor and studying the effects of its addition None of these factors is based on very sure foundations both because the interpretation of techniques used to demonstrate them is uncertain and because deduction from abnormal to normal physiology is dubious The probability of the existence of any one of these factors depends on cumulative evidence

To take as an example haemophilia For many years haemophilia was a syndrome defined mainly by the clinical features and inheritance The clotting defect was not explained by coagulation theory Then Addis (1911) showed that a fraction of normal plasma would correct the defect Addis misinterpreted his results in the light of then current knowledge but his experiments were confirmed by Govaerts and Gratia (1931) Patek and Stetson (1936) and others and a coagulation factor antihaemophilic globulin was gradually accepted. According to this work antihaemophilic globulin is a substance deficient in haemophilic blood and present in the fibrinogen fraction of normal blood Initially the factor could not be incorporated into blood coagulation theory and its demonstration rested on a very circular argument First a patient would be said to have haemophilia from the clinical features and inheritance a substance would then be said to have antihaemophilic activity if it corrected the patient s defect Much more recently (Brinkhous 1947 Quick 1947 Biggs Douglas and Macfarlane 1953) the factor was incorporated into the theory of normal physiology as a substance necessary for the formation of blood thromboplastin and an independent test for its activity based on normal physiology was proposed There is still practically no information about the chemical properties of the substance and the nature of the reactions in which it takes part

From this example four phases of definition of a clotting factor can be distinguished

(1) The recognition of a disease syndrome
(2) The identification of a deficiency

(3) The interpretation of the deficient factor in terms of normal physiology
(4) The chemical characterization of the factor and the reactions that it undergoes

At every stage of this process there are pitfalls for the unwary In the first stage many dissimilar conditions may be grouped together and causally identical cases excluded on narrow definitional grounds At the second stage also dissimilar conditions may be grouped together (e g haemophilia and Christmas disease) and confusing ideas about the nature of the deficient factor may come from using different patients at random to characterize the factor At this and later stages it may remain very difficult to distinguish between a deficiency of a coagulation factor and the presence of an inhibitor by which it is neutralized If advance is to be made it is probably wise to assume the deficiency theory in the first instance unless there is good evidence for its rejection The third stage marks an important advance because it opens the way for independent methods of study In the fourth stage the coagulation factor may be said to have emerged as a true and real substance

The factors now postulated in coagulation theory are at various stages in this evolutionary process and it is of more than academic interest to define the stages that they have reached Two factors only prothrombin and fibrinogen are well advanced in stage 4 these may be considered to be true and real substances Quite a number of factors are fairly securely established in stage 3 Factor V reached stage 3 at its first serious appearance thanks to the brilliant work of Owren (1947) Factor VII Antihaemophilic globulin and the Christmas factor are similarly placed by cumulative evidence from many sources The tissue factor and platelets may be included in this category though these factors were studied more in their relation to normal physiology than to disease states

Factors emerging from obscurity at the present time include the plasma thromboplastin antecedent (P T A) factor of Rosenthal Dreskin and Rosenthal (1953) and Factor X of Koller (1954 1955) The information about the P T A factor is confusing and nothing is known about its possible role in normal physiology on the other hand the clinical features and a pattern of laboratory findings in the patients is rather definite It would be best to consider this condition as a syndrome Koller s factor is rather different the facts about its

properties are confused by the apparent multiple defects caused by the dicoumarol drugs and no case of an isolated deficiency of this factor has been described The factor could be placed rather hesitatingly at stage 2 and referred to as a defect

We should like to propose that a convention should be adopted by which a new condition characterized mainly by clinical features and an unexplained pattern of experimental results should be referred to as a syndrome That conditions in which the blood can be shown to lack some substance replaceable by a definite fraction of normal blood should be said to have a defect and that the status of coagulation factor should be reserved for those entities which can be shown to play some part in a system composed wholly of normal reagents Real substances are those about which some definite chemical evidence is available (Prothrombin and Fibrinogen) Thus we should have the Rosenthal Syndrome (P T A deficiency) or the dicoumarol delay defect (Factor X deficiency)

THE THEORY OF BLOOD COAGULATION

Theories of blood coagulation are a necessary guide to experiment and as knowledge advances the theory changes Any theory leaves much unexplained the one which we propose as a working hypothesis Fig 26 excludes early reactions involving contact with a foreign surface about which little is known and for simplicity

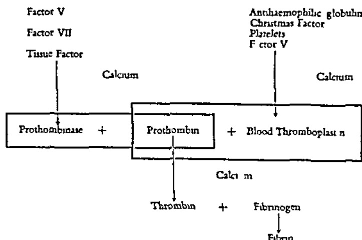


Fig 26 Diagram of a working hypothesis of blood coagul t on.

Table 20

Tissue System				Blood System			
Time Relations	*Tests*	*Factors*	*Nature of Reaction*	*Nature of Reaction*	*Factors*	*Tests*	*Time Relations*
	Proconvertin Test; Two-stage Prothrombin Reaction to Thrombin; One-stage Prothrombin time	Factor VII	The effects of contact with a foreign surface	The effect of contact with a foreign surface	Christmas Factor Platelets	Whole Blood Clotting Time; Calcium Clotting Time; Thromb-elastogram; Thrombin Generation Test; Prothrombin Consumption Test; Thromboplastin Generation Test	2–5 min
8–10 sec		Factor V Factor VII Tissue Factor *Calcium*	Prothrombinase Formation	Blood Thromboplastin Formation	Christmas Factor Platelets A H G Factor V Calcium		
2–5 sec		Prothrombin Prothrombinase and *Calcium*	Thrombin Formation	Thrombin Formation	Prothrombin Blood Thromboplastin Calcium		2–5 sec.
2–5 sec.		Fibrinogen Thrombin	Fibrin Formation	Fibrin Formation	Thrombin Fibrinogen		2–5 sec

excludes inhibitory substances The diagram fails to give any explanation for the P T A or Factor X phenomena The diagram suggests that there may be two distinct mechanisms for the production of prothrombin converting substances In the one prothrombinase is derived from tissues it must be presumed that this is a relatively subsidiary system since its normality in haemophilia does not prevent bleeding in the other the blood system forms blood thromboplastin This scheme is put forward to account for the fact that the blood system does not appear to require Factor VII which is essential for the tissue activator This is a very simple scheme merely designed as a working hypothesis for use in the study of patients for the interpretation of tests of clotting function For the latter purpose the scheme has been divided into clotting phases (Table 20) each phase involving the formation of some active coagulant.

The omission of the effects of surface contact and of the inhibitory substances means that this scheme gives little idea of the clotting process as a whole It is probable that intra-vascular fluidity is the result of a delicately balanced equilibrium the activation of precursor coagulants being favoured by roughened surfaces and the effective action of the coagulants being limited, probably by a whole series of inhibitory or neutralizing factors Coagulation will occur if the coagulant factors overcome the inhibitors and coagulation having occurred further clots may be expected, because the products of clotting are also clot promoting (thrombin Factor VII and the Christmas factor) On the other hand, depression of a coagulant or an increase in inhibitory substances will cause hypocoagulability Clotting must always depend on the formation of coagulants at rates faster than they can be neutralized, thus speed of action of a factor is just as important as the amount present 100 per cent of prothrombin in the blood will be no more effective than 1 per cent if it is converted to thrombin at 1/100 of the normal speed

THE ROLE OF CALCIUM IN BLOOD COAGULATION

Calcium appears to be required very generally in the clotting process Since there is practically no certainty about the nature of any of the clotting reactions except about the thrombin-fibrinogen reaction in pure solutions it is difficult to study the role of calcium. Another difficulty lies in the distinction between bound and ionized calcium and which of these may be active in clotting When

citrated plasma is exposed to contact with a glass surface, activation of some dissolved factor or factors (possibly the Christmas factor and Factor VII) occurs with no added calcium (Biggs Douglas and Macfarlane 1953 Rapaport et al 1954) Changes in platelet activity also occur slowly in the absence of calcium (Hougie 1955 Biggs Douglas and Macfarlane 1953) It would appear that some early reactions can take place without calcium unless sufficient is provided by that incorporated into the glass surface In addition the thrombin-fibrinogen reaction can take place without calcium Apart from these calcium appears to be necessary for all other reactions There are many enzyme systems in which the combination between a protein and metal are essential for activity It is possible that calcium in a combined form is required for the majority of blood clotting reactions as for example Bergsagel (1955) has suggested for the reactions involved in the activation of the Christmas factor and its reaction with platelets On the other hand the natural reactions involve very large thromboplastic particles which can be removed by centrifuging at 15-25 000 r p m These large particles may provide adsorbtion surfaces at which the conversion of prothrombin takes place and the provision of a suitable surface may involve the adsorbtion of calcium ions as has been suggested by Lovelock and Porterfield 1951 (see Chapter X)

TESTS OF CLOTTING EFFICIENCY

The techniques used to record clotting efficiency have evolved against a changing background of theory Many of these tests have proved useful and will continue to be used regardless of the interpretation placed on the results But the scope of the various tests can be increased if their results are interpreted in terms of modern experimental patterns old tests can be modified to give new interpretations and new techniques can be devised In the present section it is proposed to assess the significance of tests which can now be used to test clotting function These vary greatly in complexity the simplest tests are not always the easiest to interpret The majority of clotting tests record the results of reactions rather than amounts of substances though with ingenuity many can be modified to give a probable assay of individual substances As a first step the *tests can be visualized according roughly to the phase of clotting* by which they are influenced (Table 20) The tests can also

be subdivided into those in which tissue extracts are added among the reagents and those in which no tissue extracts are used It is proposed to start with the simplest and most general tests and to continue with the more specific which are also usually more complicated

GENERAL TESTS OF CLOTTING FUNCTION

THE WHOLE BLOOD CLOTTING TIME (Appendix IV 1)

This test consists in placing a measured volume of blood into each of four glass tubes and recording the time which elapses before a solid clot appears In Chapter VI it was shown that during the clotting of whole blood there is an initial lag phase of 3-4 minutes when no thrombin can be detected. This lag phase is occupied by the reactions involving contact with a foreign surface and blood thromboplastin formation. Directly any thrombin can be detected the blood clots solidly The whole blood clotting time therefore is influenced mainly by the duration of the lag phase of clotting and will give grossly abnormal results if the lag phase is long Since haemophilia is the commonest cause of a prolonged lag phase most of the patients whose blood clotting is greatly delayed have haemophilia

The method is influenced by technical variables such as the size of the syringe and needle the size of glass tube used the method of washing the tubes and the number of times that the tubes are tilted during clotting The major effects of technical factors means that the test cannot be used for recording small differences The test remains clinically useful because from our experience 99 per cent of the patients with very greatly prolonged clotting times (more than 30 min when the normal range is 5-10 min) have haemophilia The test cannot be used to assess the adequacy of treatment in haemophilia because normal results can be obtained on the blood of patients whose clotting function is grossly abnormal The test is unlikely to be very useful for detecting defects in later stages of clotting because these are normally rapid in comparison with the initial stages and gross defects will cause relatively little lengthening of the normal lag phase For example if the lag phase in a sample is taken to be 4 minutes and the next phase normally occupies 15 seconds an increase in this second phase to 1 minute may lengthen the clotting time to 5 or 6 minutes which is not significantly abnor-

mal The clotting time is probably only prolonged when the factors taking part in these later phases (prothrombin fibrinogen etc) are virtually absent

THE CALCIUM CLOTTING TIME (Appendix IV 3)

In this test citrated or oxalated plasma is mixed with sufficient calcium chloride to neutralize the anticoagulant and the clotting time is recorded The test always gives shorter clotting times than the whole blood test moreover the results are affected by the number of platelets in the plasma and by the length of time for which the plasma has stood in a glass tube the clotting time shortening progressively on exposure to glass (Hougie 1955)

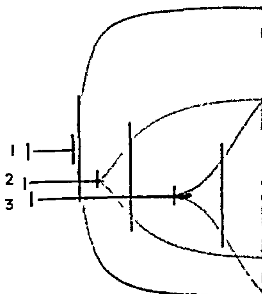
The test therefore has all the disadvantages of the whole blood clotting time with additional technical variation On the other hand it has the advantage that the sample can be collected and the tests done at leisure

THE THROMB-ELASTOGRAPH

Hartet (1950, 1951 1952 etc) has devised an apparatus intended to record the rigidity of clots formed in whole blood or plasma The apparatus records the first onset of clotting and the speed of increasing clot rigidity and the final firmness of the clot The technique provides a permanent photographic record of the results and patterns of abnormality have been recorded in different conditions Like the whole blood clotting time and the calcium clotting time the test is probably influenced by all stages of clotting and is affected by a variety of technical factors It is very difficult at present to say exactly what the method measures but the patterns of results are undoubtedly interesting and the method might very well be useful in experimental work on specific clotting defects Examples of the results of the test are given in Fig 27

THE THROMBIN GENERATION TEST (Appendix IV 27)

Macfarlane and Biggs (1953) devised a simple method for recording the amount of thrombin formed at intervals during the clotting of whole blood In the test 2 ml of whole blood are placed in a tube and incubated at 37 C and 0 1 ml amounts are removed at intervals and added to fibrinogen the clotting time of the fibrinogen samples giving a record of the amount of thrombin present A curve relating amount of thrombin and time can then be drawn



Fg 27 Thromb-el tog m records of pl san ples f o 1 n al subject a d from two p t ents u der t tn nt f r c ronary th ombosis. R co d (1) is from subject und r t e t me t w th dinde an wh se o -st ge proth on b n t me w s 22 sec (normal = 15 sec) The curv would be sa d to d cate a co tinu g thrombot c tendency R co d (2) is f o n m l subject Re d (3) is fro n a second p tient under treatment with dindev n whose on -st ge proth mb n t me was 38 sec (nor al = 15 sec) Th record shows reduction n co gulab l ty when c mp rison is made w th the n rm l This record w kindly s ppl ed by Dr Eth l B dwell

The curves are characteristically abnormal in many coagulation defects and it has been found that definitely abnormal results are found in the blood of patients who are mildly affected clinically The test has the disadvantage that the results cannot be expressed quantitatively Pitney and Dacie (1953) have described a modification of this test in which plasma is used

All of these tests are non-specific in that no special phase of clotting is emphasized but in all the phase of longest duration (the effects of surface contact and blood thromboplastin formation) are the most important In the two simplest tests the whole blood clotting time and the calcium clotting time technical variability reduces the usefulness of the results which are in addition insensitive to any but very large deviations from normal The other tests give much more information the thromb-elastogram gives new information but at present it is difficult to interpret the test may be rather insensitive to some clotting defects we have obtained normal results on the blood of one quite severely affected haemophilic patient The thrombin generation test gives easily interpretable information and the test is sensitive to small changes Biggs Douglas and Macfarlane (1953) have used this test to study the effects of glass contact on clotting Though all of the tests are non-specific they may be modified, using the blood of patients with known clotting defects to give specific information Thus the effects of material thought to have antihaemophilic globulin activity may be tested by making additions of the material to haemophilic blood and recording the results of one or other of these tests on the mixtures

TESTS OF BLOOD THROMBOPLASTIN FORMATION

There are two tests in which the early stages of clotting are separated from the phases involving the conversion of prothrombin to thrombin and fibrin formation One test, the prothrombin consumption test includes the earliest stage of clotting the effects of glass contact but gives non-specific information The other test the thromboplastin generation test, excludes the earliest stages but gives more specific results

THE PROTHROMBIN CONSUMPTION TEST (Appendix IV 2)

During normal clotting blood thromboplastin converts practically all of the prothrombin to thrombin which is then neutralized in

THE THROMBOPLASTIN GENERATION TEST (Appendix IV 28)

In this test three reagents are prepared from normal blood these are platelets and plasma treated with $Al(OH)_3$ (or $BaSO_4$ Duckert et al 1954) and serum All of these reagents have been exposed to contact and thus the earliest stages of clotting are not taken into account The test records blood thromboplastin formation only In the test the three blood reagents are mixed with $CaCl_2$ and incubated at 37 C At minute intervals 0 1 ml of the incubated mixture is added together with 0 1 ml of $CaCl_2$ to 0 1 ml amounts of citrated plasma The clotting times of the plasma samples which are recorded give a measure of the amount of thromboplastin formed in the incubation mixture

The technique involving the preparation of three reagents has rather large technical variation but the differences from normal obtained with pathological specimens are so large that this technical variation is unimportant For diagnosis one or other of the normal reagents is substituted by a corresponding reagent from the patient (see Table 21) Thus if haemophilic $Al(OH)_3$ treated plasma is substituted for the normal reagent (Mixture 2 Table 21) the

TABLE 21

Mixture	*Source of Reagents*			*Conditions which give normal results*
	$Al(OH)_3$ treated plasma	*Serum*	*Platelets*	
1	Normal	Normal	Normal	Control test done in all experiments
2	Patient	Normal	Normal	Haemophilia Factor V deficiency*
3	Normal	Patient	Normal	Christmas disease
4	Normal	Normal	Patient	Thrombasthenia may give abnormal results
5	Patient	Normal	Patient	Factor V deficiency
6	Patient	Patient	Normal	Rosenthal's syndrome (called P T A. deficiency)

* The results may be nearly normal in Factor V deficiency because Factor V is supplied with the normal platelets.

serum by antithrombin If blood thromboplastin is not formed much prothrombin remains in the serum after coagulation The prothrombin consumption test consists in allowing a measured volume of blood to clot in a glass tube and at a specified time after clotting (usually 1 hour) the prothrombin activity of the serum is tested In most techniques no attempt is made to assay the serum prothrombin specifically, as assessed from clotting times serum samples may be said to contain as much as 2 or 3 × the amount of prothrombin present in the original blood Such high figures are almost certainly due to the presence of clot accelerating factors (Factor VII and intermediate products of thromboplastin *formation*) If an attempt is made to assay the prothrombin specifically these high figures are not obtained (Douglas and Biggs 1953)

The test records a deficiency of thromboplastin formation but the cause of the deficiency remains obscure The test is complementary to the thrombin generation test which measures the amount and speed of thrombin formation during clotting If there is gross delay or much reduced thrombin formation much prothrombin will remain in the serum Since it is usual to record the results at one time only there is a definite threshold below which abnormality will not be recorded The test is therefore less sensitive than the thrombin generation test Since whole blood is used the earliest stages of clotting will be recorded

This test is useful because it is simple to carry out and gives abnormal results with a wide variety of clotting defects It is influenced by the same technical variables which affect the whole blood clotting time thus the results may be difficult to standardize In our laboratory the results have changed gradually over a period of five years presumably from slight changes in glass tubes syringes needles and methods of washing glass etc To obtain exact results normal samples must be tested frequently and the results on patients blood compared with a current normal series Though non-specific the test may be modified to give specific information using known pathological blood Thus Graham et al (1950) have devised a method for measuring antihaemophilic activity using the blood from haemophilic dogs to which additions are made of material thought to have antihaemophilic activity The results can be compared quantitatively with some standard preparation The method is satisfactory but requires a constant supply of haemophilic blood.

THE THROMBOPLASTIN GENERATION TEST (Appendix IV 28)

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5	Patient	Normal	Patient	Factor V deficiency
6	Patient	Patient	Normal	Rosenthal's syndrome (called P T A. deficiency)

* The results may be nearly normal in Factor V deficiency because Factor V is supplied with the normal platelets.

results are abnormal and if Christmas disease serum is used the results are also abnormal (Mixture 3 Table 21) If mixtures 2 and 3 both give abnormal results the presence of a circulating anticoagulant must be suspected this may be tested for by preparing mixture 1 and adding some of the patient s $Al(OH)_3$ treated plasma, a depression of thromboplastin formation indicates the presence of an inhibitor in the patient s $Al(OH)_3$ plasma. If no inhibitor can be detected the possibility of a *combined deficiency of Christmas* factor and antihaemophilic globulin must be borne in mind. An example of the use of this test is shown in Table 22 The results in this table are given as *clotting times but they may also be expressed* as thromboplastin concentrations using the curve of Fig 19 for the conversion

TABLE 22

Condition Tested	*Source of Reagents*			*Incubation Time Minutes* / *Clotting time seconds*				
	Al(OH)₃ Plas	*Serum*	*Platelets*	1	2	3	4	6
Normal	Normal	Normal	Normal	77	60	27	11	11
Haemophilia	Patient Normal	Normal Patient	Normal Normal	70 31	62 17	65 12	61 12	48 12
Christmas Disease	Patient Normal	Normal Patient	Normal Normal	70 65	54 45	30 36	15 39	10 36
Circulating Anticoagulant	Normal Patient	Patient Normal	Normal Normal	115 64	91 41	75 51	60 67	70 59
Rosenthal s Syndrome (P T A Deficiency)	Normal Patient Patient	Patient Normal Patient	Normal Normal Normal	45 53 60	27 33 44	15 21 32	12 14 24	11 13 14

Occasionally patients are encountered who have very slight deficiencies and the difference from normal is not great In these cases the results may be made more definite by diluting the appropriate reagent with a sample from a patient of known *deficiency* An example of this modification of the test is shown in Table 23 The Christmas disease patient included in the table is an extremely mildly affected *patient in whom the diagnosis still remains somewhat* in doubt though other evidence (clinical and family history) is suggestive

TABLE 23

Suspected Defect	Source of Reagents: $Al(OH)_3$ Plasma	Serum	Platelets	Incubation Time Minutes 1 (Clotting time seconds)	2	3	4	6
Normal	Normal	Normal	Normal	58	17	10	12	11
Haemophilia	Patient	Normal	Normal	62	49	13	14	12
	Haemophilia 9 Patient 1	Normal	Normal	67	58	40	31	24
	Haemophilia 9 Normal 1	Normal	Normal	40	20	13	14	14
Christmas Disease	Normal	*Patient	Normal	45	42	17	11	10
	Normal	Christmas 19 Patient 1	Normal	55	36	14	15	15
	Normal	Christmas 19 Normal 1	Normal	55	16	12	10	11

* The figures indicate the proportions of reagents used, the amounts of the mixtures included are the same in all tests.

The thromboplastin generation test has now been used to establish the diagnosis in patients from more than 100 families Doubtful results were obtained in only five instances (three patients with probable haemophilia and two with probable Christmas disease) Using the clotting time and prothrombin consumption test twenty case gave entirely normal results despite a very definite clinical history of bleeding The thromboplastin generation test is therefore superior to other tests for the diagnosis of thromboplastin defects not only does it give specific information it is also more sensitive to slight abnormalities

THE ANTIHAEMOPHILIC GLOBULIN ASSAY METHOD (Appendix IV 30e)
The thromboplastin generation test has the disadvantage that, in its original form it cannot be used to give a quantitative measure of any particular clotting factor Any attempt to prepare antihaemophilic globulin for administration to haemophilic subjects or to assess the effects of treatment requires a reliable assay method Since normal results in the thromboplastin generation test are dependent on antihaemophilic globulin it is possible to devise a method of assay based on this test (Biggs Eveling and Richards 1955)

In the thromboplastin generation test all the reagents necessary for thromboplastin formation are incubated together and samples are removed and tested for their ability to accelerate the clotting of

recalcified plasma It is found that after incubation a minimum clotting time is reached The amount of antihaemophilic globulin present influences the minimum clotting time low levels producing longer clotting times In the assay method the reagents are incubated for 10–25 minutes (the exact time depending on the reactivity of the reagents) and the clotting time of the substrate plasma is recorded The test is carried out with doubling dilutions of the antihaemophilic material to be tested and the results compared with those of a standard preparation

The method has been used in in vitro experiments in parallel with other tests of clotting function (prothrombin consumption test, thrombin generation test and clotting time) and all of the tests appear to give a parallel record of abnormality The test records relatively minor deviations from normal and has been used in the control of therapy bleeding being very greatly reduced or normal in amount after operations if the level of antihaemophilic globulin is above 30 per cent of normal In in vivo experiments the results of the assay method and the prothrombin consumption test are not well correlated

THE CHRISTMAS FACTOR ASSAY

Pitney (1955) has devised a similar method for the assay of the Christmas factor The method has the disadvantage that it makes use of known Christmas disease serum which may not be readily available

TESTS IN WHICH TISSUE EXTRACTS ARE USED

When tissue extracts are added to recalcified plasma the earliest stages of clotting are certainly excluded Though tissue extracts may not correspond exactly with any product of the blood system they are probably roughly equivalent to one of the intermediate products From the practical point of view tissue extracts replace the Christmas factor antihaemophilic globulin and platelets or some product of reaction between these substances The remaining substances Factor V Factor VII and Prothrombin will affect tests carried out using brain extracts

THE ONE-STAGE PROTHROMBIN TIME (Appendix IV, 7)

In this test 0 1 ml. of citrated or oxalated plasma are mixed with 0 1 ml of tissue extract and 0 1 ml of $CaCl_2$ is added and the

clotting time is recorded. The clotting time will be lengthened if Factor V Factor VII or prothrombin is reduced It should be noted (Biggs and Douglas 1953 Hunter and Walker 1954) that the test is apparently very insensitive to prothrombin deficiency giving normal results in samples quite grossly deficient in prothrombin The test will also give abnormal results if fibrinogen is reduced or if an inhibitor of the thrombin fibrinogen reaction (such as heparin) is present

MODIFICATIONS OF THE ONE-STAGE PROTHROMBIN TIME USING DILUTED PLASMA

As originally described the one-stage prothrombin time is usually carried out on undiluted plasma Using this method the clotting time is little affected until the constituent to be measured is below 10 per cent of normal Link (1944) and Brambel (1950) noted that small deviations from normal were more obvious if the test were carried out on diluted plasma The simple dilution techniques have the disadvantage that fibrinogen is also reduced and the test is unreliable physiological variations in fibrinogen content affecting the results Owren and Aas (1951) devised a dilution method in which fibrinogen and Factor V are supplied at high concentration (Appendix IV 8) The method will record abnormalities of Factor VII and prothrombin and since these two factors are commonly reduced together the test is useful It is said to be much more reliable for the routine control of anticoagulant therapy with the dicoumarin group of drugs than the unmodified test (Owren 1954b and c Astrup and Müllertz 1954) Preliminary experiments in this laboratory appear to confirm this claim The only disadvantage to the modified method is that it is technically rather more difficult

MODIFICATIONS OF THE ONE-STAGE PROTHROMBIN TIME AND THE MEASUREMENT OF FACTORS V AND VII

The test may be modified to give more precise information by supplying a constant high concentration of one or other of the factors which affect the test Thus if Factor V fibrinogen and prothrombin are supplied the test may be used to measure Factor VII quantitatively (Owren and Aas 1951, Koller Loeliger and Ducket 1951) Similarly if Factor VII and prothrombin are supplied the test may be used to measure Factor V

THE ONE-STAGE PROTHROMBIN TIME AND THE MEASUREMENT OF PROTHROMBIN

If an excess of Factors V and VII is added to the reagents for the one-stage test the method may be used to measure prothrombin (Owren and Aas 1951) In practice this method is not very satisfactory because alterations in the concentration of prothrombin do not greatly influence the clotting time

Hjort, Rapaport and Owren (1955) have devised a method using Russell s viper venom Since the test using Russell s viper venom is not affected by the concentration of Factor VII the only reagent to be supplied is Factor V The test appears from the results of Hjort et al to be very satisfactory The method seems to give rather lower results for prothrombin concentration when used on the blood of patients receiving the dicoumarin anticoagulants than does the two-stage method described below The reason for this discrepancy is obscure

THE TWO-STAGE METHOD FOR THE MEASUREMENT OF PROTHROMBIN (Appendix IV 14)

In the two-stage test brain emulsion and calcium are added to plasma and this mixture is called the incubation mixture At known time intervals after the addition of calcium samples are removed from the incubation mixture in which thrombin is forming and added to fibrinogen The clotting times of the fibrinogen samples are recorded The clotting time of the fibrinogen gives a measure of the amount of free thrombin present in the incubation mixture when the sample was removed An example of the results of this test carried out on normal plasma is shown in Table 24 and Fig 28 From this example it will be seen that the clotting times of

TABLE 24

THE RESULTS OF A TWO-STAGE PROTHROMBIN TEST ON NORMAL PLASMA

	Incubation time in minutes						
	$\frac{1}{4}$	$\frac{3}{4}$	1	2	3	4	5
Clotting time seconds	20	17	18	31	77	170	180+
Thrombin units per ml. of incubation mixture	6.2	7	6 8	3 5	1.2	0.2	0

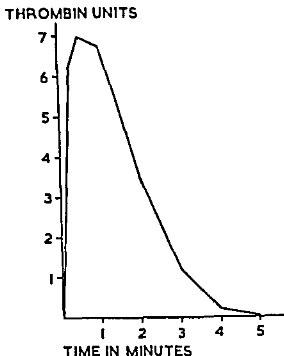


Fig 28 Thrombin formation in normal plasma tested by the two-stage method This curve represents the a erage results of 15 normal samples

successive samples shorten until a minimum is reached and after this lengthen again because the thrombin is neutralized by antithrombin It is probable that the two reactions the formation of thrombin and its neutralization proceed together from the start The peak level of thrombin formed occurs at a point where most of the prothrombin has been converted to thrombin and the rate of formation of thrombin is balanced by its neutralization The question that arises is how can a curve of this sort be used to measure prothrombin? It is usual to assume that the peak value of thrombin formed is proportional to the amount of prothrombin present Thus the test is carried out on normal and pathological plasma and the two peaks are compared. The height of the peak in the pathological plasma is expressed as a percentage of that in the normal plasma It is clear that if one unit of prothrombin is converted to

THE ONE-STAGE PROTHROMBIN TIME AND THE MEASUREMENT OF PROTHROMBIN

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THE RESULTS OF A TWO-STAGE PROTHROMBIN TEST ON NORMAL PLASMA

	Incubation time in minutes						
	¼	½	1	2	3	4	5
Clotting time seconds	20	17	18	31	77	170	180+
Thrombin units per ml. of incubation mixture	6 2	7	6 8	3 5	1 2	0 2	0

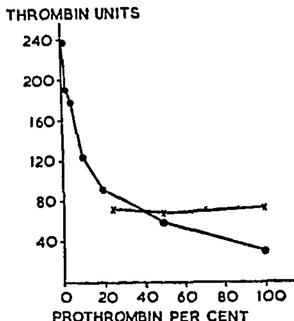


Fig 29 The two-stage test was carried out on saline dilutions of normal plasma. The amount of thrombin (read from a dilution curve) represented by the minimum clotting time given by the tests was recorded. The amount of thrombin was multiplied by the dilution to give the theoretical thrombin forming capacity of the plasma The results of this test are shown ●—● It will be seen that the apparent thrombin forming capacity of the plasma increases with dilution. The results of a similar test using Factor V and prothrombin in place of plasma are shown x—x

for measuring prothrombin A would represent prothrombin, B thrombin and C a neutralized thrombin-antithrombin association In the two-stage method it is B which is measured The speed of formation of B will depend on the presence of activating substances and the rate of destruction of B will depend on the concentration of the neutralizing factor

When the amount of A is varied but the speeds of formation and neutralization of B are unchanged the curves shown in Fig 30b are obtained In these curves the peak value of B is proportional to the amount of A initially present and the area enclosed by the curves will give a proportionate measure of A

When the amount of A is constant and the speed of neutralization of B is unchanged variation in the speed of formation of B gives

one unit of thrombin the level of thrombin reached in units cannot be the same as the initial amount of prothrombin The peak level of thrombin is probably reached before all of the prothrombin has been converted to thrombin and by this time an unknown proportion of the thrombin has already been neutralized If the peak level of thrombin is not equal to the initial concentration of prothrombin is it proportional to this level? The peak level of thrombin may be proportional to the amount of thrombin formed but only in very closely defined conditions To understand this problem it is necessary to study the effect of antithrombin in this reaction more closely

The complicating effect of antithrombin in the two-stage method was well recognized by those who proposed the methods (Warner Brinkhous and Smith 1936 Herbert 1930 etc) and they suggested that the plasma to be tested by the two-stage method should be greatly diluted to remove its effects A simple experiment will show that dilution does not solve the difficulty A number of dilutions of plasma are made with 0 85 per cent saline and these are tested by the two-stage method The amount of thrombin present when the peak value is reached can be calculated for each dilution If these thrombin levels represent the amount of prothrombin present in the original plasma then multiplication by the dilution factor for each dilution should give a constant level of thrombin per ml of plasma. In fact the level of thrombin calculated increases with increasing dilution and there is no tendency for the curve to flatten at the higher dilutions (Fig 29) When a purified preparation of prothrombin is tested by this method the calculated level of prothrombin remains approximately constant The curve shown in Fig 29 represents dilutions from 1/3 to 1/300 dilution greater than 1/300 cannot be tested by this method because the fibrinogen clotting times exceed 60 seconds and become unreliable Thus for practical use of the test the effects of antithrombin must be taken into account

To visualize the type of reaction involved in the formation of thrombin in normal plasma it is convenient to consider a simplified theoretical model of the reaction Suppose that a substance A is converted to another substance B by a first order reaction and substance B is simultaneously converted to C by a first order reaction (Fig 30a) If this reaction is followed the concentration of A falls and that of B increases as the reaction proceeds As B is formed it is converted to C Thus a peak level in the formation of B is reached when most of A has been converted to B In the two-stage method

curves such as those shown in Fig 30c The peak value of B reached is no longer a measure of the amount of A initially present but the area enclosed by the curves is proportional to the amount of A present at the beginning From the diagrams in Fig 30 it is clear that the constancy of the areas is due to the slower disappearance of B Thus if C is constant the slower speed of formation of B can be detected by the slower disappearance of B In plasma samples with delayed thrombin formation the slow speed of thrombin disappearance may be more obvious than the slow formation of thrombin

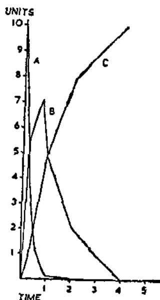
The variation in the level of thrombin achieved which results from delay in thrombin formation was recognized by Owren (1947) and Ware and Seegers (1949) These authors therefore recommend that in the two-stage method an excess of conversion factors should be added These modifications of the two-stage test undoubtedly increase its reliability but in practice they are far from simple it is difficult to know how much of the accelerator substances to add and the results will be affected by the presence of antithrombin

When the amount of A is constant and the speed of formation of B is unchanged but the speed of neutralization of B is varied then curves such as those shown in Fig 30d are obtained In these circumstances neither the peak level of B nor the areas enclosed by the curves can give a proportional measure of A

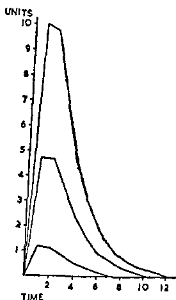
Following this theoretical model it is possible to see conditions which must be fulfilled before the unmodified two-stage method can be used to give a proportional measure of prothrombin. The most generally applicable measure of prothrombin will be obtained by calculating the area enclosed by the two-stage curves First it is important to know how closely the thrombin generation curves of the two-stage method approximate to the theoretical model In Fig 31 a theoretical curve has been fitted to the curve obtained as an average for fifteen samples of normal plasma. It will be seen that the correspondence is remarkable

The amount of prothrombin can be varied by adding prothrombin (which contains an excess of Factor VII) to prothrombin deficient plasma When this is done the curves are similar in pattern to those of Fig 30 (Fig 32)

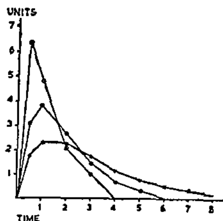
The speed of thrombin formation can be varied by the use of the plasma of a patient under treatment with tromexan When this is



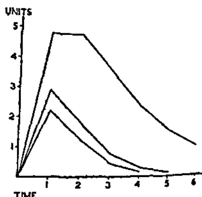
(a)



(b)



(c)



(d)

Fig 30. Calculated curves for a theoretical reaction in which substance A is converted to B by a first order reaction and simultaneously B is converted to C.

( ) Curves to show the disappearance of A and the formation of B and C

(*b*) The concentrations of B at different times when formed from 3 different initial concentrations of A

(*c*) The concentrations of B at different times assuming three different speeds of formation at B The initial concentration of A and the speed of neutralization of B are constant.

(*d*) Curves to show the effect on the concentration of B of varying the speed of conversion of B to C the initial amount of A and the speed of formation of B being constant

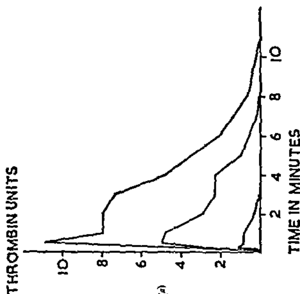


Fig 32 Experimental observations in which the amount of prothrombin in plasma was varied compared to the theoretical curves.

(*a*) Experimental results Different amounts of prothrombin prepared by adsorption were added to plasma of a patient with uncomplicated prothombin deficiency and thrombin formation was followed by the two-st ge method.

(*b*) Theoretical curves

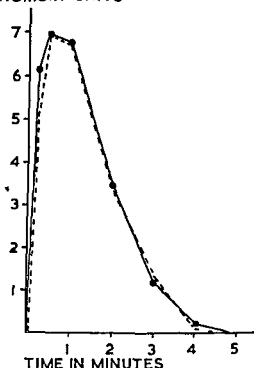
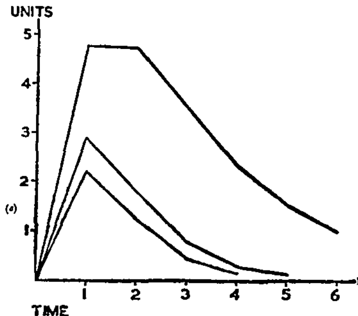


Fig 31 Thrombin formation in normal plasma tested by the two-stage method (average results of 15 samples) compared to a theoretical curve The continuous line represents the experimental results and the discontinuous line the theoretical curve

done the curves of Fig 33 are obtained These are of the same general pattern as those of Fig 30

The speed of thrombin neutralization can be varied by the addition of heparin When this is done the curves of Fig 34 are obtained These are of the same pattern as Fig 30

From these tests it is probable that the generation of thrombin in the two-stage method approximates sufficiently closely to the theoretical model for the application of deductions from this model to the measurement of prothrombin The measurement of prothrombin based on deductions from the theoretical curves is likely at least to be better than a measurement of prothrombin based on assumptions that are certainly false



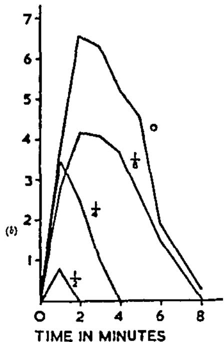


Fig 34. Experimental observations in which the speed of thrombin neutralization was varied compared to the theoretical curves.
(a) Theoretical results.
(b) Experimental results Varying amounts of heparin (0 1/8 1/4 and 1/2 unit) were added to normal plasma and thrombin formation was followed by the two-stage method.

THROMBIN UNITS

7 6 5 4 3 2 1

(a)

0 1 2 3 4 5 6 7

TIME IN MINUTES

UNITS

7 6 5 4 3 2 1

(b)

1 2 3 4 5 6 7 8

TIME

Experimental observations in which the speed of thrombin formation was varied compared to the theoretical curves

(a) Experimental results. x—x Thrombin formation in tromexan plasma O—O Thrombin formation in tromexan plasma to which 10 per cent of normal serum was added ●—● Thrombin formation in normal plasma.

(b) Theoretical curves.

TABLE 25

THE LEVELS OF VARIOUS CLOTTING FACTORS LIKELY TO RECORD A SIGNIFICANT ABNORMALITY BY VARIOUS TESTS OF CLOTTING EFFICIENCY

Factor	*TESTS*						
	Whole Blood Clotting Time	*Prothrombin Consumption Test*	*Thrombin Generation Test*	*Thromboplastin Generation Test*	*AHG Assay Method*	*One-stage Prothrombin Time*	*Two-stage Prothrombin Time*
Fibrinogen	0-200 mg/100 ml	Not affected	Not affected	Not affected	Not affected	0-100 mg/100 ml.	Not affected
Prothrombin	0-10 per cent	Not affected	Low level of thrombin	Not affected	Not affected	0-10 per cent	0-100 per cent
Factor V	0-5 per cent	? 0-5 per cent	? Low and delayed	? 0-20 per cent	Not affected	0-10 per cent	Not affected
Factor VII	Not affected	Not affected	Not affected	Not affected	Not affected	0-5 per cent	Not affected
Anti-haemophilic globulin	0-3 per cent	0-10 per cent	0-10 per cent	0-20 per cent	0-50 per cent	Not affected	Not affected
Christmas Factor	0-3 per cent	0-10 per cent	0-10 per cent	0-20 per cent	Not affected	Not affected	Not affected
Platelets	Not affected	0-50 000 c.mm	0-100 000 c.mm.	Unsuitable test	Not affected	Not affected	Not affected

The correspondence between the observed and theoretical curves does not necessarily mean that the reactions of blood clotting follow this simple pattern Other explanations may be equally valid or other reactions may take place too fast to influence the general pattern From the close approximation to the theoretical model all that can be deduced is that provided the conditions for making the test are not altered the area method of measuring prothrombin is likely to give more generally reliable results than other methods

In practice the two-stage test is carried out on both normal and pathological plasma samples The results of the tests are plotted as curves to show the generation of thrombin (Fig 28) and the area enclosed by the two curves is calculated The area obtained with the pathological specimen is then expressed as a percentage of the normal area (Appendix IV 14) This method is not only more generally applicable than other methods but it is likely to be more reliable In methods which depend on determining peak values of thrombin it is obviously important to determine the peak reliably Since this peak level is often of very short duration this is not simple By the area method the exact determination of the peak level of thrombin is relatively unimportant

TWO-STAGE METHODS IN WHICH ANTITHROMBIN IS DESTROYED

Sternberger (1947) devised a method in which 25 per cent of ethyl alcohol is added to the two-stage incubation mixture The method has the disadvantage that the presence of the alcohol prevents the normal clotting of fibrinogen and clotting times have to be determined from the appearance of granularity in the solution Fantl (1954) has devised a method using pyrocatechol in place of alcohol The pyrocatechol does not interfere with the clotting of fibrinogen and the method might prove satisfactory though preliminary tests suggest that only some of the antithrombin is destroyed by pyrocatechol It has so far not been tested in parallel with other methods

CONCLUSION

No attempt has been made to create a comprehensive theory of blood coagulation a theory has been proposed merely to facilitate the study of the better recognized coagulation defects New cases are always a challenge to ingenuity and in studying these cases it

PART TWO

must always be remembered that the theory may be entirely wrong Faced with such a new case it is necessary to hesitate before creating a new factor and to obtain a clear idea of the actual amount of information available If a true deficiency has been demonstrated by the correction of the defect by a specific fraction of normal plasma then the condition may be named as a new defect If such information is not available then the case is merely 'an unusual case or if many such cases are known a 'syndrome Only if there is clear information about the role of the possible substance in normal physiology can a new factor be named

In Table 25 is given a summary of the levels of different factors likely to affect tests of clotting function This table gives the range of levels at which a significant change from normal is likely to be recorded From this table it is possible to get some idea about the relative usefulness of the tests in different deficiencies



(a)



(b)

PLATE 2

( ) Electron m croscope photog ph of normal pl telets gold sh dow ng techn que from Bes (1950)
(b) Elect n m croscope phot g ph of norm l pl telet by B unste r F b r nd Klein (1950)

THE DISORDERS OF BLOOD COAGULATION

In the previous section of this book observations and theories on the mechanism of blood coagulation have been discussed from an academic and experimental point of view The chapters which follow deal mainly with the practical consequences of disordered coagulation that is with facts that many doctors have to face in their clinical work when they are called upon to treat cases of abnormal bleeding or of thrombosis

Before the subject of abnormal bleeding can be discussed it is necessary to consider the capabilities of the normal defences against haemorrhage It is a strange fact that the haemostatic mechanism as a whole has received very little attention probably because it is so unobtrusively efficient that most people are hardly aware of its existence It is essentially concerned with the control of bleeding from the small vessels the capillaries and venules and arterioles which are liable to be damaged by such trivial everyday procedures as brushing the teeth and more obviously by the scratches knocks and cuts which everyone sustains It is a common and unnoticed experience that such injuries stop bleeding of their own accord though it is recognized that bleeding from larger vessels usually requires some mechanical or surgical haemostatic treatment Even the surgeon seems genuinely to believe that in applying a few clamps and ligatures to his operation wound he has made the major contribution towards haemostasis He forgets that nature has quietly sealed the countless small vessels that he has also divided and that in the absence of this normal haemostatic process all his ordinary precautions against haemorrhage would be useless It is a sad fact that failure to appreciate this dependence upon nature has led to a number of deaths following surgery optimistically undertaken in cases of haemorrhagic disease

As often happens with unobtrusive efficiency it is only when the haemostatic mechanism fails that the importance of its normal working is fully appreciated Its failure means to the patient that a normal life becomes almost impossible and that he may die at any time of bleeding from some small injury Many such cases of abnormal

bleeding are directly due to defective blood coagulation though the precise way in which clotting is concerned in haemostasis is not yet established and it is certain only that its action is less simple than would appear at first sight It is now known for example that clotting defects which may result in severe bleeding are not always demonstrable by methods for estimating the simple coagulation time Thrombin formation may be seriously impaired and the haemostatic efficiency of coagulation may be grossly deficient without any obvious delay in the time at which the first strands of fibrin appear

As clinical entities the haemorrhagic states were known long before the importance of blood coagulation or the existence of platelets were recognized Cases of haemophilia and purpura were described centuries ago but the deficient coagulation or reduction of platelets in these conditions was only demonstrated within the last sixty or seventy years It is less than twenty-five years since the abnormal bleeding in cases of jaundice and of haemorrhagic disease of the new-born was shown to be due to defective coagulation Within the last ten years it has been shown that specific haemorrhagic states are due to deficiencies of Factor V Factor VII or Christmas factor or to the presence of naturally occurring inhibitors Nature has, in fact provided a series of controlled experiments in which each of the factors known to be concerned in coagulation are separately reduced Only a deficiency of calcium is missing a reduction of calcium sufficient to inhibit coagulation occurs in the textbooks but not apparently in real life We have seen cases of severe hypocalcaemic tetany in which there was no demonstrable impairment of coagulation

A deficiency of the platelets (thrombocytopenia) is often associated with haemorrhage The ways in which the platelets may participate in haemostasis have been a matter of dispute for a considerable time some authorities believe that they agglutinate to form haemostatic plugs which seal off damaged vessels others consider that they are directly or indirectly concerned with vascular contraction There is no doubt that the platelets are concerned in blood coagulation and that a reduction in their number causes a reduction of the amount of thrombin formed and of the contractility of the clot produced However platelet deficiency is usually associated with a type of bleeding which is different from the bleeding observed in cases of defective coagulation without thrombocytopenia In the coagula-

tion disorders haemorrhage occurs from or into any part of the body usually as a result of trauma and the formation of massive deep-tissue haemorrhages is characteristic In the thrombocytopenic states bleeding tends to be superficial taking the form of petechiae or ecchymoses in the skin or oozing from the mucus membranes which may occur apparently spontaneously Though the platelet deficiency diseases are included among the disorders of coagulation it is unlikely that the clotting defect produced by thrombocytopenia is the major cause of the haemorrhage There is no doubt that in such cases there are changes in the capillaries and it is probable that defective capillary function is an important cause of the abnormal bleeding A discussion of this aspect is clearly beyond the scope of this book

The naturally occurring specific deficiencies of the various clotting factors provide unique opportunities for the study of their action with the least manipulation of the plasma. Many investigators have taken brilliant advantage of these opportunities so that much of the knowledge of normal coagulation has been directly derived from the study of the abnormal In return the experimenter from the knowledge he has gained has been able to provide the clinician with the means to treat his patients The discovery of Vitamin K which has abolished a whole group of haemorrhagic disorders is one of the major advances in medicine The recognition of anti-haemophilic globulin has given at least a palliative treatment for haemophilia. The local use of efficient coagulants and absorbable dressings is of practical help in many surgical and clinical situations The growing knowledge of coagulation has also increased the precision of diagnosis in the haemorrhagic states and has made possible technical methods by which hitherto unrecognized defects in the clotting mechanism can be detected

If the haemorrhagic diatheses have received considerable attention it is because they are essentially dramatic conditions often associated with an obvious and impressive struggle for the life of the patient By contrast, intravascular thrombosis is a less spectacular condition, which has received comparatively little attention and the small space allotted to it in the following chapters is an index of the lack of available experimental material Yet from the clinical point of view thrombosis is a far more important problem than abnormal bleeding A patient may live for many years despite a total failure of his clotting mechanism but the coagulation of the blood in

bleeding are directly due to defective blood coagulation though the precise way in which clotting is concerned in haemostasis is not yet established and it is certain only that its action is less simple than would appear at first sight It is now known for example that clotting defects which may result in severe bleeding are not always demonstrable by methods for estimating the simple coagulation time Thrombin formation may be seriously impaired and the haemostatic efficiency of coagulation may be grossly deficient without any obvious delay in the time at which the first strands of fibrin appear

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certain vessels is incompatible with life Even small and localized areas of thrombosis in the heart or brain are frequently fatal and many more people die of thrombosis than of abnormal bleeding Though anticoagulant therapy is rapidly increasing in extent and the mode of action and clinical efficiency of the anticoagulant drugs are being studied with increasing energy knowledge of the basic causes of thrombosis is still almost non-existent It is not known for instance if intravascular coagulation is primarily due to a general increase of the coagulability of the blood or to changes in the vascular endothelium or to changes in the conditions of the circulation It is probable that the growing knowledge of the factors concerned in coagulation will make available new methods for investigating these problems Relatively crude methods have not demonstrated any constant and significant increase in the coagulability of the blood in cases of thrombosis but a careful assay of factors (such as those concerned with thromboplastin generation) which have been appreciated only recently may give valuable information

CHAPTER XIII

FIBRINOGEN DEFICIENCY

A deficiency of fibrinogen is a very rare cause of defective coagulation but when it does occur it may produce a severe haemorrhagic diathesis An apparently total and permanent lack of fibrinogen may exist as a congenital defect a chronic deficiency may occur as an acquired condition in various states in which fibrinogen production may be defective and it is now recognized that an acute fibrinogen deficiency may develop with disastrous rapidity in certain obstetrical and surgical cases The minimum blood fibrinogen level required for effective clotting is not easy to define in uncomplicated afibrinogenaemia it seems that as little as 60 mgs per cent is enough to restore apparently normal clotting in acute obstetrical cases abnormal bleeding may occur when the blood level of fibrinogen is 100 mgs per cent or more but in such cases other clotting factors may also be deficient.

CONGENITAL ABSENCE OF FIBRINOGEN

An apparently complete absence of fibrinogen occurring as a congenital defect has now been described in about twenty cases including those of Rabe and Saloman (1920) Opitz and Frei (1921) Macfarlane (1938b) Henderson Donaldson and Scarborough (1945) Pinniger and Prunty (1946) Revol and Favre-Gilly (1947) Prentice (1951) Albeggiani and Grutta (1954) and Caussade et al (1954) The descriptions give a fairly definite picture of a haemorrhagic diathesis resembling haemophilia but affecting either sex In several instances two or more brothers or sisters have been similarly affected Though there is no definite evidence that the condition is hereditary it is significant that in at least six families the parents of the affected children were cousins and in three instances abnormally low blood fibrinogen levels were found in the parents though they had no abnormal bleeding (Macfarlane 1938b Lawson 1953 Caussade et al 1954) It is probable therefore that congenital afibrinogenaemia is due to a simple recessive character which when it occurs in the homozygous form as the result of chance or intermarriage causes severe abnormality

CLINICAL AND LABORATORY FINDINGS

Though the haemorrhagic tendency is similar to that of haemophilia with the same persistent bleeding from small injuries and into the joints and tissues it is a remarkable fact that cases of afibrinogenaemia are much less severely affected than the average haemophilic patient Fatal bleeding from the umbilical cord which may have been due to afibrinogenaemia has been reported in a few siblings Opitz and Frei (1921) record a case of fatal bleeding from a cut finger but in most of the other cases bleeding has been more troublesome than dangerous The residual disabilities which effect the joints or haematoma sites in haemophilia seem not to occur and even menstruation may be normal (Lawson 1953) The reasons for this relative mildness in spite of the completely incoagulable blood in such cases are obscure and raise doubts as to the precise function of fibrin formation in haemostasis It should be remembered that in fibrinopenia the clotting mechanism is normal apart from fibrinogen so that platelet metamorphosis and thrombin production will proceed normally whereas in haemophilia they will not The lack of damage to tissues following bleeding may itself be due to the lack of fibrin formation and subsequent fibrosis

The principal laboratory finding in afibrinogenaemia is the complete absence of clotting by any of the usual tests If both the Lee and White and the Quick 1-stage prothrombin time tests show no clotting or a very incomplete clot then total or partial fibrinogen deficiency should be suspected Absence of fibrinogen is confirmed by failure of the citrated blood or plasma to clot on adding thrombin and by the absence of a precipitate on heating the plasma to 56 C for 10 minutes or on adding to the plasma an equal volume of 50 per cent saturated solution of ammonium sulphate Studies of the earlier stages of the clotting mechanism have shown that thrombin generation and thus probably thromboplastin generation is normal (Hardisty and Pinniger 1956) Alexander et al (1954) have found that following contact with glass afibrinogenaemic blood shows the normal agglutination and lysis of its platelets and the normal consumption of A H G Factor V and prothrombin

In a few cases thrombocytopenia has been reported with a lengthening of the bleeding time (Macfarlane 1938b Glanzmann 1939 Glanzmann et al 1940) but in the majority there was no abnormality of this type The fibrinogen deficiency seems to be due to a specific failure of production since no abnormal utilization or destruction

of fibrinogen could be demonstrated No other demonstrable abnormality of plasma proteins or liver function in these cases has been reported

EFFECTS OF REPLACEMENT THERAPY

The temporary replacement of fibrinogen by blood transfusion or the injection of fibrinogen preparations has been useful both clinically and in the study of the fate of fibrinogen in the circulation Breckoff (1924) studying Rabe and Salomon s case found that blood transfusion restored normal coagulation and that detectable fibrinogen persisted in the circulation for ten to seventeen days Pinniger and Prunty (1946) confirmed that transfused fibrinogen was still detectable after 8 days and that a blood level of 20-30 mgms per cent was sufficient to cause the formation of apparently normal clots showing good retraction but that about 60 mg per cent is required for effective haemostasis (Pinniger and Prunty 1946 Stefanini and Petillo 1949) Gitlin and Borges (1953) followed by immunological methods the survival of injected fibrinogen and found that 50 per cent is lost in 48 hours with a subsequent logarithmic rate of decrease It seems from these studies that the haemostatic defect can be temporarily corrected by giving fibrinogen and since it persists for several days after injection there is no rapid destruction of fibrinogen in these congenital cases It has been reported, however that antibodies to transfused fibrinogen may be developed (Brönnimann 1954)

CONSTITUTIONAL FIBRINOPENIA

There is also a less well defined condition termed by Risak (1935) constitutional fibrinopenia He describes four patients all women who were said to have reduced amounts of fibrinogen but in each case more than 100 mg per cent was present. There was a moderate reduction of the platelets in two of these patients and all of them had a tendency to abnormal bruising and bleeding from small abrasions In two instances there was a similar condition in other members of the family Similar cases are described by Allibone and Baar (1943) and Heinild (1944)

ACQUIRED FIBRINOGEN DEFICIENCY

Acquired fibrinogen deficiency is much more common than the congenital condition It is brought about by diseases which impair

fibrinogen production, or by the rapid utilization of fibrinogen caused by widespread clotting in vivo or its destruction by rapid fibrinolysis in the circulation The deficiency caused by impaired production is usually incomplete and may not be sufficiently severe to cause bleeding Risak (1935) refers to malignant disease infections and diseases involving the bone marrow as reducing the fibrinogen content of the blood Opitz and Silberberg (1924) report a case of absence of fibrinogen in a child suffering from tuberculosis of the spleen and liver Jurgens and Trautwein (1930) described a man, aged 52 with absence of fibrinogen and a severe haemorrhagic diathesis following the widespread destruction of the bone marrow caused by carcinomatosis Wolff (1936) observed a severe haemorrhagic diathesis in a child aged five weeks with a reduced blood fibrinogen probably due to congenital syphilis

Lowering of the blood fibrinogen level may also occur in patients with impaired absorption from the gastro-intestinal tract (Yeager Rhoads and Freeman 1947) and in other nutritional conditions infections and liver diseases (Ham and Curtis 1938 Frodin 1947 and Dyggve 1947) Bjorkman (1948) describes cases of polycythaemia, pernicious anaemia and leukaemia in which the fibrinogen level of the blood was reduced In most of these cases the reduction is insufficient to cause abnormal haemorrhage A comprehensive review of congenital and acquired deficiency of fibrinogen with further examples of the latter condition has been published by Favre-Gilly (1947)

Fibrinogen deficiency may occur usually terminally in conditions in which liver function is grossly impaired such as acute phosphorous or chloroform poisoning and acute yellow atrophy of the liver due to other causes Whipple and Hurwitz (1911) showed that chloroform poisoning sufficient to induce central necrosis of the liver in dogs resulted in a fall in blood fibrinogen to almost zero With regeneration of liver tissue the fibrinogen was gradually restored to normal In these experiments it must be remembered that chloroform also may cause active fibrinolysis and *it is possible that the* fibrinopenia may be due to destruction of fibrinogen rather than to impaired production by the liver Nolf and his co-workers have for many years investigated the effect of extirpation of the liver on the blood coagulation mechanism and a constant feature of their experiments is a reduction in the fibrinogen In his most recent publication (Nolf and Adant 1950) typical experiments are described

in which after extirpation of the liver an injection of peptone produces first of all a failure of the clotted blood to retract and secondly a progressive fall in blood fibrinogen to total disappearance In these experiments active fibrinolysis was noted so that again the supposed decreased production by the liver is complicated by increased destruction in the circulation

'ACUTE DEFIBRINATION SYNDROME

An excessive utilization or destruction of fibrinogen in vivo may produce a dangerous haemorrhagic state with fulminating suddenness and since it involves factors other than fibrinogen is referred to here as the 'acute defibrination syndrome

This condition which has only recently received general recognition occurs in association with pregnancy and as a complication of certain surgical operations As a complication of pregnancy it has been described by Dieckmann (1936) Molony Egan and Gorman (1949) Weiner et al (1950) Schneider (1951) Seegers and Schneider (1951) Favre-Gilly (1952) Reid (1953) Moore (1954) and many others It may arise shortly before or during labour or abortion and may produce within a few hours fatal bleeding from the uterus or a generalized haemorrhagic state The blood is usually found to be incoagulable and tests will show absence or a great reduction of fibrinogen In some cases intense fibrinolytic activity has been described incomplete clots being formed and then rapidly lysed or the patient s blood being capable of lysing added fibrin Fibrinogen deficiency is usually not the only abnormality though the findings vary with the stage of development of the condition There is often thrombocytopenia and a deficiency of prothrombin Factor V and A H.G (Greenwalt and Triantaphyllopoulus 1954) The cause of the condition is the subject of much discussion It is almost always associated with obstetrical abnormalities involving necrosis or premature separation of the placenta and is thus seen in cases of abruptio placentae placenta praevia concealed accidental haemorrhage eclamptic or pre-eclamptic toxaemia and intra-uterine foetal death Dieckmann (1936) suggested that massive retro-placental haemorrhage might deplete the circulating blood of fibrinogen. A more likely explanation is that of Schneider (1951) who suggested that, in the conditions mentioned, fragments of necrotic placental tissue became detached and entered the maternal circulation through the large open blood spaces at the placental site

Such tissue fragments are known to be actively thromboplastic *and their entry into the blood stream would probably cause paradoxically*, incoagulability of the blood by a process known as the 'negative phase reaction Many of the earlier workers on coagulation observed that incoagulability could be produced by the slow intravenous injection of thromboplastic tissue extracts or other substances capable of promoting clotting such as cream chloroform serum or silica (Pickering 1928) Mellanby (1909) showed that coagulant viperine snake venoms injected slowly caused more or less complete defibrination of the circulating blood Nolf (1922a) believed that this was due to slow and generalized intravascular coagulation which produced not thrombosis of large vessels but the laying down of a thin layer of fibrin on the internal surfaces Such fibrin deposits are likely to be rapidly lysed since fibrinolytic activity is usually increased by the procedures employed Zucker (1948) found that intravenous injection of thrombin in dogs produced defibrination in vivo and also a prolongation of the prothrombin time and Hartmann Conley and Krevans (1951) observed that the intravenous injection of large amounts of brain thromboplastin in dogs caused rapid death from wide-spread thrombosis, but the injection of smaller amounts caused decreased coagulability of the blood with *fibrinopenia thrombocytopenia*, and prolongation of the prothrombin time

There is thus good evidence for supposing that *if tissue thromboplastin* in the form of placental fragments were to gain access to the blood stream the effects observed in the defibrination syndrome might be produced Seegers and Schneider (1952) have produced experimental evidence that such placental emboli do occur and it is *possible that in some cases amniotic fluid may enter the blood* stream and produce effects similar to those of tissue thromboplastin (Reid Weiner and Roby 1953) The intense fibrinolysis observed in some cases is also considered to be a cause of the fibrinogen deficiency It is by no means a constant feature (Masure and Schockaert 1954) and was absent in a severe case associated with foetal death recently observed by us When present, fibrinolysis *might be due to activators of the fibrinolytic system* derived from placental tissue or amniotic fluid (Astrup 1956)

A very similar syndrome has been observed as a complication of certain surgical operations Most of the reported cases have occurred during or shortly after pulmonary lobectomy Soulier et al (1952)

investigated a series of eight fatal cases finding afibrinogenaemia and active fibrinolysis It is possible that the passage of thromboplastic lung tissue fragments into the circulation may occur during such operations, and produce effects similar to those already described Thyroidectomy has also been associated with the defibrination syndrome (Lhoiry and Fayet 1954)

Diagnosis The occurrence of unexpectedly profuse or uncontrollable bleeding in any of the obstetrical or surgical situations mentioned should at once suggest the possibility of acute defibrination syndrome In the early stages bleeding may be limited to the areas of trauma but as the condition progresses generalized bleeding in the form of echymoses bruising and spontaneous haemorrhage from the mucus membranes may develop The blood is incoagulable or forms frail clots that may rapidly lyse The prothrombin time test may show no clotting or the delayed formation of a clot obviously less solid than normal The usual tests for fibrinogen will show complete absence or considerable reduction The addition of fibrinogen in vitro to freshly drawn blood samples may not result in coagulation since other clotting factors such as Factor V or A H G may be deficient. There is frequently severe or complete thrombocytopenia and the patient may have a prolonged bleeding time and a positive tourniquet test.

Because of the urgency in most cases these tests are often of more academic interest than of value to the clinician and some rapid and simple indication of fibrinogen deficiency is essential Schneider (1952) makes serial dilutions of whole blood with saline and observes the highest dilution at which observable clotting takes place in comparison with a normal blood sample treated in the same way Weiner Reid and Roby (1950) simply observed the behaviour of a whole blood sample In fibrinopenia clotting either does not occur at all or a very frail clot forms or the clot appears and then lyses within an hour A rapid and simple quantitative test for fibrinogen may be employed, this consists of adding an equal volume of 1/2 saturated ammonium sulphate to the patient s plasma and measuring the volume of the precipitate in a special graduated tube after centrifuging comparing the result with a normal control test (Appendix IV 17b)

Treatment Since the patient s blood lacks fibrinogen and usually other clotting factors it might be supposed that the rapid transfusion of normal blood would give the best results It is stated that in this

Such tissue fragments are known to be actively thromboplastic, and their entry into the blood stream would probably cause paradoxically incoagulability of the blood by a process known as the 'negative phase' reaction Many of the earlier workers on coagulation observed that incoagulability could be produced by the slow intravenous injection of thromboplastic tissue extracts or other substances capable of promoting clotting such as cream chloroform serum or silica (Pickering 1928) Mellanby (1909) showed that coagulant viperine snake venoms injected slowly caused more or less complete defibrination of the circulating blood Nolf (1922a) believed that this was due to slow and generalized intravascular coagulation which produced not thrombosis of large vessels but the laying down of a thin layer of fibrin on the internal surfaces Such fibrin deposits are likely to be rapidly lysed since fibrinolytic activity is usually increased by the procedures employed Zucker (1948) found that intravenous injection of thrombin in dogs produced defibrination in vivo and also a prolongation of the prothrombin time and Hartmann Conley and Krevans (1951) observed that the intravenous injection of large amounts of brain thromboplastin in dogs caused rapid death from wide-spread thrombosis but the injection of smaller amounts caused decreased coagulability of the blood with fibrinopenia thrombocytopenia and prolongation of the prothrombin time

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A very similar syndrome has been observed as a complication of certain surgical operations Most of the reported cases have occurred during or shortly after pulmonary lobectomy Soulier et al (195-)

HYPOPROTHROMBINAEMIA INCLUDING FACTOR V FACTOR VII AND PROTHROMBIN DEFICIENCIES

The concept of prothrombin deficiency arose from the classical theory of blood coagulation. According to this theory the one- and two-stage prothrombin tests carried out on plasma give a quantitative measure of prothrombin and patients whose plasma gives abnormal results by these tests would be said to have hypoprothrombinaemia in fact the one-stage test measures deficiencies in Factors V and VII and is affected by fibrinopenia and the presence of inhibitory substances Since this early era in blood coagulation research the problem has been complicated by several new techniques and conceptions It is now certain that the majority of patients whose plasma samples give a long one-stage prothrombin time have not got true prothrombin deficiency It is possible to obtain more information about the patients using modifications of the one-stage prothrombin time in which various additions are made to the plasma Thus a long one-stage clotting time may be reduced by the addition of normal serum or by normal plasma treated by Al(OH) $BaSO_4$ etc (see Chapter V) Two types of defect may be separated in this way the cases in which correction is obtained with normal serum being said to have Factor VII deficiency and those in which correction was achieved with adsorbed plasma being said to have Factor V deficiency The two-stage tests which usually give some idea of true prothrombin deficiency were also done and a new pattern of single and combined deficiencies began to emerge Recently cases have also been studied using the thromboplastin generation test and difficulties have arisen in the interpretation of the results

The most simple approach to the problem is to describe advances in knowledge historically taking first advances based on acceptance of the classical theory of blood coagulation. The advances made using modifications of the one-stage test designed to distinguish between Factor V and Factor VII deficiency forms the next stage of development The most recent advances are provided by a study of blood thromboplastin formation

condition a blood level of fibrinogen below 100 mg per cent is associated with defective clotting (Weiner et al 1953) and rational treatment should presumably aim at restoring a level higher than this For this purpose 4–5 pints of blood should theoretically be sufficient, particularly since the patient s blood volume is likely to be depleted It has been reported however that transfusion of these amounts of blood may fail to correct bleeding or to have any obvious effect on the clotting process (Moore 1954) In contrast it has been reported that the injection of fibrinogen is effective and it is surprising that apparently good results have been obtained with relatively small amounts Moore (1954) for instance from his own experience and from a survey of the literature believes that 2 gm of fibrinogen may be sufficient and some other observers have claimed good results with even less (Barnett and Cussen 1954) It is at present difficult to understand why this amount of fibrinogen should be beneficial when the much larger amounts present in the blood and plasma transfused was apparently ineffective It must be remembered that it is actually very difficult to assess the evidence for the effect of treatment in this condition since blood transfusions had also been given to the patients who responded well to fibrinogen injection and there is usually no time or justification for withholding one form of therapy in order to judge the effect of the other

Summary

Fibrinogen may be absent from the blood as a congenital defect Usually such patients are the children from a cousin marriage The coagulation defect is clinically less severe than is usual in haemophilic patients Transfused fibrinogen is detectable in the blood of these cases for 8 days

Fibrinopenia may occur as the result of some other diseases such as acute infections carcinoma gastro-intestinal malabsorbtion liver disease etc Acute depletion of fibrinogen referred to as the acute defibrination syndrome may occur as a complication of delivery and operations It is thought that the defect is due either to acute fibrinolysis or intravascular coagulation

quinone had Vitamin K activity It was then shown that a large number of compounds with related structure were active

During this work the methods for the estimation of prothrombin were being studied and patients with various diseases were investigated with these new techniques to determine the clinical incidence of hypoprothrombinaemia It was found that in biliary obstruction a disease long known to be associated with haemorrhage there was often a profound fall in plasma prothrombin Quick (1937) suggested that the deficient absorption of fat in the intestine might interfere with the absorption of Vitamin K and that the hypoprothrombinaemia in these patients might be due to Vitamin K deficiency The demonstration in very numerous studies that Vitamin K caused a rapid fall in the prothrombin time in patients with obstructive jaundice supported Quick s hypothesis (Warner et al. 1938 Butt et al 1938a and b Olsen and Menzel 1939 Smith et al 1939 etc)

In patients with steatorrhoea or prolonged diarrhoea the absorption of Vitamin K from the gut may be reduced These patients may have a haemorrhagic diathesis which can be cured by parenteral Vitamin K

One or two cases have been recorded in which Vitamin K deficiency arose in otherwise apparently normal people and gave rise to a severe haemorrhagic diathesis (Lewis and Bennet 1947 Heindl et al 1948 Ferguson 1950) Two cases with a severe deficiency of Vitamin K have also been described by Ley et al (1951) These last two patients are particularly interesting because their coagulation defects were not cured by the synthetic water soluble Vitamin K analogue but the naturally occurring Vitamin K_1 was effective Thus if the water soluble Vitamin K analogue is ineffective in an obscure case of prothrombin deficiency it is always advisable to give the naturally occurring oil soluble Vitamin K_1 Quick and Collentine (1951) have shown that Vitamin K_1 is more effective than the water soluble preparations in restoring the prothrombin level of animals with experimental Vitamin K deficiency

Acute injury to the liver such as that caused by chloroform poisoning is associated with a fall in prothrombin as tested by the two-stage method (Smith Warner and Brinkhous 1937) The prothrombin level is often not corrected by Vitamin K and may indicate severe liver damage In cirrhosis of the liver a prothrombin deficiency which is not corrected by Vitamin K is common

EARLY ADVANCES IN THE PHYSIOLOGY OF 'HYPOPROTHROMBINAEMIA'

Initially the discovery that some plasma samples had a long one-stage prothrombin time led to the study of haemorrhagic syndromes using the new test It was soon found that plasma samples from haemophilic patients gave normal results but in some other conditions the results were abnormal This stage of investigation will be discussed under a number of headings

(1) Vitamin K deficiency and gross liver disease
(2) Haemorrhagic disease of the newborn
(3) Dicoumarin poisoning
(4) Congenital deficiencies

Vitamin K Deficiency and Liver Disease

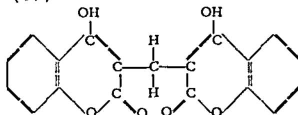
The discovery of Vitamin K followed investigation of a haemorrhagic disease of chicks which was caused by feeding purified diets This disease was initially thought to be due to Vitamin C deficiency because it was cured by feeding green plants It was soon shown that ascorbic acid did not cure the disease and that extraction of the diet with ether removed the curative substance Dam (1934) and Dam and Schønheyder (1935) suggested that the chicks lacked a new fat soluble vitamin Vitamin K (Koagulation Vitamin)

The disease was found to be associated with a long whole blood clotting time (McFarlane Graham and Richardson 1931, Dam and Schønheyder 1935) and the addition of crude chick prothrombin made by Howell s or Mellanby s method corrected the defect (Dam Schønheyder and Tage-Hansen 1936)

Initially it was found that the haemorrhagic chick disease appeared rather inconsistently even if the chicks were given identical diets It was then observed that Vitamin K appeared in the diet if it underwent bacterial fermentation (Halbrook 1935) and that the droppings of chicks contained Vitamin K so that the diet might become contaminated (Almquist and Stockstad 1935 1936) It was assumed that Vitamin K was synthesized in the intestine but that this synthesis occurred too low down in the gut for absorption to take place

The isolation of substances with Vitamin K activity followed many independent investigations Dam and Schønheyder (1936) and Klose et al (1938) studied the properties of preparations having Vitamin K activity Concentration of Vitamin K was achieved by Almquist (1936) Riegel et al (1939) and Dam et al (1939) In 1939 Almquist and Klose showed that 2-methyl-3-hydroxy 1 4-naphtho-

tury and it was suspected that the disease was a haemorrhagic septicaemia In 1924 Schofield traced the disorder to eating spoiled sweet clover Roderick (1931) showed that when prothrombin prepared by Howell s method was added to the plasma of diseased animals the clotting time was reduced and using Howell s technique Roderick was unable to prepare prothrombin from the blood of affected animals Later Quick s one-stage prothrombin technique was found to give abnormal results with the plasma of diseased animals An anticoagulant substance was isolated by Campbell and Link (1941) and synthesized by Stahman Huebner and Link (1941) and Huebner and Link (1941) Its chemical formula was thus established



Using the purified substance 3 3 Methylene-bis-(4 hydroxy-coumarin) Link (1944) showed that the degree of hypoprothrombinaemia was directly related to the amount of the drug administered

Dicoumarin and related drugs are used widely for the treatment of patients with thrombosis and it has been found in practice that the one-stage prothrombin test or some modification of this test is the most reliable guide to therapy Using this method haemorrhage may be avoided if the clotting time is kept between fairly defined limits This problem is discussed in detail in Chapter XVIII

Congenital Hypoprothrombinaemia

A number of cases recorded in Table 26 suffered from a haemorrhagic diathesis from early infancy In three of the six studies there was a history of haemorrhage in other members of the family These cases are probably similar to others which have been studied in greater detail and which will be considered later

DEFICIENCY OF FACTORS V AND VII AND PROTHROMBIN DETERMINED USING MODIFICATIONS OF ONE- AND TWO-STAGE TESTS

From a knowledge of the properties of Factors V VII and pro-

Haemorrhagic Disease of the Newborn

Before the introduction of Vitamin K a tendency to haemorrhage was seen in approximately 1 in every 400 newly-born babies Bleeding occurred from the mucous membranes umbilicus or genito-urinary tract and haematemesis and melaena were common In a review of 60 cases Capon (1924 1932 1937) described the dramatic onset of haemorrhage *in otherwise healthy infants* The average time of onset in these cases was 41 5 hours after delivery and haemorrhage usually occurred before the fifth or sixth day In Capon's series the death rate was 8 2 per cent Examined at post-mortem ulceration of the gastro-intestinal tract was found but these lesions were thought to be the effect rather than the cause of haemorrhage

Rodda (1920) and Beveridge (1928) found that the infants had a long whole blood clotting time In 1937 Brinkhous Smith and Warner *found that the prothrombin tested by the two-stage method* was low at birth and remained low for many months Owen et al (1939) and Quick and Grossman (1939a b 1940) on the other hand found that by the one-stage method the prothrombin level was low for a very limited period in the first ten days of life when haemorrhage occurred This discrepancy between the two methods foreshadowed the difficulties which were later to arise from the study of accelerators of blood coagulation *The discrepancy has* never been explained because the blood of new-born infants has *never been examined systematically* by modern methods

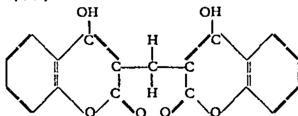
Before knowledge of Vitamin K was available the disease was treated by local styptics by the intramuscular injection of whole blood or serum or by transfusion The only effective treatment was transfusion

In 1939 Waddell et al showed that the lengthened one-stage prothrombin time of infants was reduced by Vitamin K and if the Vitamin K was given early the hypoprothrombinaemia could be *prevented These findings which have been confirmed* by very numerous workers provide a means not only of treating the disease but of preventing its occurrence It is thought that the deficiency in Vitamin K results from a failure of absorption from the gastro-intestinal tract in the early days of life

The Coagulation Defect in Dicoumarin Poisoning

A *haemorrhagic* disease of Canadian cattle associated with a long whole blood clotting time was recognized early in this cen-

tury and it was suspected that the disease was a haemorrhagic septicaemia In 19_4 Schofield traced the disorder to eating spoiled sweet clover Roderick (1931) showed that when prothrombin prepared by Howell s method was added to the plasma of diseased animals the clotting time was reduced and using Howell s technique Roderick was unable to prepare prothrombin from the blood of affected animals Later Quick's one-stage prothrombin technique was found to give abnormal results with the plasma of diseased animals An anticoagulant substance was isolated by Campbell and Link (1941) and synthesized by Stahman Huebner and Link (1941) and Huebner and Link (1941) Its chemical formula was thus established



Using the purified substance 3 3 Methylene-bis-(4 hydroxy-coumarin) Link (1944) showed that the degree of hypoprothrombinaemia was directly related to the amount of the drug administered

Dicoumarin and related drugs are used widely for the treatment of patients with thrombosis and it has been found in practice that the one-stage prothrombin test or some modification of this test is the most reliable guide to therapy Using this method haemorrhage may be avoided if the clotting time is kept between fairly defined limits This problem is discussed in detail in Chapter XVIII

Congenital Hypoprothrombinaemia

A number of cases recorded in Table _6 suffered from a haemorrhagic diathesis from early infancy In three of the six studies there was a history of haemorrhage in other members of the family These cases are probably similar to others which have been studied in greater detail and which will be considered later

DEFICIENCY OF FACTORS V AND VII AND PROTHROMBIN DETERMINED USING MODIFICATIONS OF ONE- AND TWO-STAGE TESTS

From a knowledge of the properties of Factors V VII and pro-

TABLE 26

CASES OF UNDEFINED HYPOPROTHROMBINAEMIA

Author	*Family history*	*One-stage prothrombin time*	
		Normal	*Patient*
		secs	secs
Rhoads and Fitzhugh (1941)	—	23	70-107
Plum (1943) 1	—	18	30 5
2	—	18	28
Murphy and Clark (1944)	+	16	68-92
de Marvel and Bomchil (1944)	—	Prothrombin Index 25 53 per cent	
Hauser (1945)	one family	Prothrombin Index	
1	+	21 80 per cent	
2	+	53 per cent	
Covey et al (1950)	one family		
1	+	16-25	31 100
2	+	24	41
3	+	25	78
4	+	18	31

TABLE 27

REAGENTS FOR THE QUALITATIVE DISTINCTION BETWEEN PROTHROMBIN FACTOR V AND FACTOR VII DEFICIENCY

Reagent	*Factors present*		
	Prothrombin	*Fact r V*	*Factor VII*
$Al(OH)_3$ treated plasma	—	+	—
Normal serum	—	±	+
Plasma from anticoagulant treated cases	+	+	—
Oxalated stored plasma	+	—	+
Purified prothrombin	+	—	+
Normal plasma	+	+	+

thrombin (see Chapter V) simple tests can be devised to distinguish between the coagulation defects classed as hypoprothrombinaemia Reagents freed from one or more of the three main factors can be prepared as shown in Table 27 Thus plasma treated with $Al(OH)_3$ lacks Factor VII and prothrombin If 10 per cent of this adsorbed plasma is added to a sample which lacks Factor V the clotting time by the one-stage method will be reduced almost to normal whereas the clotting time of samples lacking prothrombin or Factor VII will be little altered In a similar way the other reagents may be used In this way a rough qualitative distinction between the various coagulation defects is possible The six reagents given in Table 27 are included because various authors have preferred one or other of the reagents to distinguish between deficiency in various factors Using $Al(OH)_3$ or $BaSO_4$ treated plasma normal serum and whole normal plasma the main distinctions can be made Using these and similar tests deficiency of the various factors has been studied

FACTOR V DEFICIENCY

Liver Disease and Factor V Deficiency

Owren (1949) and Stefanini (1950a) showed that patients with severe liver disease lacked Factor V and Owren held that a reduction in Factor V indicated a poor prognosis The two patients described by Koller et al (1950) had a marked reduction in Factor V associated with haemorrhagic scarlet fever It is possible that this reduction in Factor V may have been due to liver damage caused by the severe infection Brambel (1950) and Stefanini (1951) found a reduction in Factor V in patients with leukaemia and Stefanini thought that this reduction might have been caused by liver dysfunction from massive infiltration of the liver with leukaemic cells

Factor V is not reduced in obstructive jaundice (Owren 1949 Stefanini 1950)

Post-operative Deficiency of Factor V

Stefanini (1951) found Factor V (labile factor) deficiency in patients undergoing operations The deficiency was at a maximum at about the third post-operative day and the Factor V had returned to normal by the ninth day

Carcinoma and Factor V Deficiency

Stefanini (1951) and Cosgriff and Leifer (1952) record Factor V deficiency in patients with advanced carcinoma The patient

TABLE 26

CASES OF UNDEFINED HYPOPROTHROMBINAEMIA

Author	*Family history*	*One-stage prothrombin time*	
		Normal	*Patient*
		secs	secs
Rhoads and Fitzhugh (1941)	—	23	70–107
Plum (1943) 1	—	18	30 5
2	—	18	28
Murphy and Clark (1944)	+	16	68–92
de Marvel and Bomchil (1944)	—	Prothrombin Index 25 53 per cent	
Hauser (1945)	one family	Prothrombin Index	
1	+	21–80 per cent	
2	+	53 per cent	
Covey et al (1950)	one family		
1	+	16–25	31 100
2	+	24	41
3	+	25	78
4	+	18	31

TABLE 27

REAGENTS FOR THE QUALITATIVE DISTINCTION BETWEEN PROTHROMBIN FACTOR V AND FACTOR VII DEFICIENCY

Reagent	*Factors present*		
	Prothrombin	*Factor V*	*Factor VII*
Al(OH) treated plasma	—	+	—
Normal serum	—	±	+
Plasma from anticoagulant treated cases	+	+	—
Oxalated stored plasma	+	—	+
Purified prothrombin	+	—	+
Normal plasma	+	+	+

ant of these cases is the patient described by Owren (1947) because it was from Owren s careful analysis of the clotting defect that Factor V was first established as an essential clotting factor

Owren s patient was a female aged 29 who had a history of bleeding episodes from the age of 3½ There was no history of bleeding in other members of the family The abnormal findings were a whole blood clotting time of 70 minutes and a one-stage prothrombin' time of 79-80 seconds where normal plasma clotted in 12-20 seconds The abnormality was not affected by Vitamin K In vitro the clotting time was restored to normal by 10 per cent of ox plasma treated with $Al(OH)_3$ Owren isolated the normal plasma component the deficiency of which was responsible for the patient s coagulation defect and showed that in the absence of this factor the normal conversion of prothrombin to thrombin was delayed

The other cases given in Table 28 were less fully investigated but there is little doubt that the coagulation defect was similar to that in Owren s case In three of the cases described the bleeding tendency occurred in more than one member of the family The patient described by Koller (1954) was also found to have antihaemophilic globulin deficiency

THE MEASUREMENT OF FACTOR V

A method for recording the extent of Factor V deficiency based on the one-stage technique can easily be devised (Owren and Aas 1951) The test for Factor V is similar to the test for Factor VII which has been worked out in detail because plasma deficient in Factor VII is now readily available

FACTOR VII DEFICIENCY

Dicoumarin and Tromexan Therapy

Plasma samples from patients treated with any of the coumarin drugs have a long clotting time by the one-stage method of Quick. Fahey and Olwin (1948) and Owren (1950a b) have shown that Factor V is not reduced the clotting time by the one-stage method is not reduced by adding 10 per cent of $Al(OH)_3$ treated plasma The one-stage clotting time is shortened by 10 per cent of normal serum and from the discussion in Chapter V it is clear that the plasma of patients treated with these drugs lacks Factor VII

described by Cosgriff and Leifer had carcinoma of the prostate with multiple secondary deposits and the Factor V deficiency caused a severe haemorrhagic diathesis

Congenital Factor V Deficiency

A number of cases of congenital Factor V deficiency have been described and these are summarized in Table 28 The most import

TABLE 28

CONGENITAL FACTOR V DEFICIENCY

Author	*One Stage Prothrombin time*		*Clotting Time*	*Prothrombin Consumption*
	Normal	*Patient*		
Owren (1947)	15 70	75	25	Abnormal
Frank et al (1950)	12	80	25	Abnormal
de Vries et al (1951)	13 14	-0- 2	7 14	Abnormal
Stohlman et al (1951)	15	23	14	Abnormal
Brink and Kingsley (1952)	10 5	41	14	—
Alexander and Goldstein (1952)		45	30-45	Abnormal
Bergsagel (1955)	15	43	37	Abnormal
Oeri et al (1954)		Long	Long	Abnormal
Lewis and Ferguson (1955)	Level of proaccelerin = 0		39	Abnormal
Sacks et al (1955)	—	110	25 58	Abnormal
Horder (1955)	14 0		12	Abnormal
Henri et al. (1954) 1	15	34	330	—
2		63	270	

is understandable The reduction in a coagulation factor to 50 per cent of its normal value has little effect on the one-stage clotting time The main deficiency in tromexan plasma is therefore one of Factor VII Many other substances are now used in anticoagulant therapy (see Chapter XVIII) and it must be borne in mind that the exact effects of most of these drugs is not known They may differ in detail from dicoumarin and tromexan It must also be realized that different methods for measuring prothrombin were used by different authors and all other methods give lower results than the area method

Vitamin K Deficiency and Factor VII

Patients with obstructive jaundice or steatorrhoea do not lack Factor V (Owren 1949) but the one-stage clotting time is shortened by the addition of serum The shortening of the clotting time caused by serum is less marked than that which occurs in the plasma of patients treated with dicoumarin or tromexan The effect of serum suggests that there is a reduction in Factor VII in the plasma of these patients

Congenital Factor VII Deficiency

There are now reports on 21 patients whose plasma has given a long one-stage prothrombin time which is reduced nearly to normal

TABLE 29

CONGENITAL 'FACTOR VII' DEFICIENCY

Author	*One-Stage Prothrombin*		*Whole Blood Clotting Time*	*Prothrombin Consumption*	*Clinical Bleeding*
	Normal	*Patient*			
Giordano (1943)	28	210		—	Epistaxis dental extraction
Crocket et al (1949)	14	66	11 48	—	
Landwehr et al. (1950)	12	70	12	Normal	Rectal bleeding (infant)
Alexander et al (1951)	17	72	14 17	Normal	Melaena epistaxis
Owren (1952)	14	55–60	14	Normal	Epistaxis bruising haemarthroses.
Beaumont and Bernard (1952)	?	68–205	20	Abnormal	Severe epistaxis, bruises petechiae gastro-intestinal

When patients are treated with dicoumarin it appears that prothrombin and Factor VII are both considerably reduced (Owren 1951) When tromexan is used (Biggs and Douglas 1952) the one-stage clotting time is often restored to normal by the presence of 10 per cent of serum suggesting that prothrombin is not so markedly deficient in these patients This belief is supported by the measurement of prothrombin by the area method (Chapter XI) By this

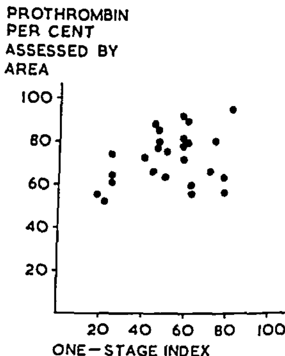


Fig 35 The prothrombin index of the one-stage test is compared with the amount of prothrombin assessed by the two-stage test in 26 samples of plasma from patients receiving tromexan.

method the amount of prothrombin in the plasma of patients treated with tromexan was seldom less than 50 per cent of normal even when the one-stage clotting time was grossly prolonged (Douglas 1955) When the results of the one-stage method are compared with the corresponding prothrombin levels no correlation is found (Fig 35) If it is accepted that the prothrombin level of tromexan plasma lies between 50 and 100 per cent of normal then this lack of correspondence of the one and two-stage tests

by the addition of a small proportion of normal serum All of these patients (see Table 29) would be said to have Factor VII deficiency It will be seen from Table 29 that many of these patients have been described very recently suggesting that the condition may not be so rare as hitherto imagined Some of the patients have been studied using the thromboplastin generation test and these results will be discussed later

THE MEASUREMENT OF FACTOR VII ACTIVITY

Factor VII may readily be measured using the one-stage method if a supply of naturally occurring Factor VII deficient plasma is avail-

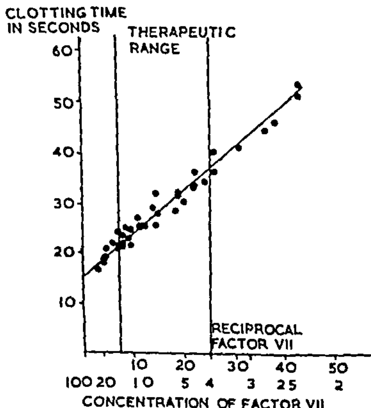


Fig 36 One-stage dilution curve of normal plasma dilutions made with tromexan plasm The results are drawn from many different experiments. The concentration of Factor VII (in the normal plasma) is plotted as a reciprocal The prob ble therapeutic range of clotting times for anticoagulant therapy is shown.

Author	One Stage Prothrombin		Whole Blood Clotting Time	Prothrombin Consumption	Clinical Bleeding
	Normal	Patient			
Lewis et al (1953)	?	73 110 48 55	13 28 30–88	Normal ? Normal	Epistaxis, haematuria, menorrhagia.
Frick and Hagan (1953)	15	67	31	Normal	Bruising
Wurzet et al (1954)	12	17	Normal	Slightly delayed	None
Jenkins (1954a)	15 17	39–70	5 8	Normal	Malaena epistaxis haemarthroses.
Hicks (1955)	13	45	6	Normal	Epistaxis, dental extraction, menorrhagia
Jurgens (1955)	Factor VII Level 2–6%		Normal	Normal	Epistaxis dental extraction.
Stefanovic et al (1953) (1) (2)	10 13	26 27	6 13	Normal Normal	*Severe epistaxis* Bruising epistaxis.
De Vries et al (1955) (1) (2) (3)	15 12 8 14 7	20 17 19 8		— — Abnormal	All severely affected with haemarthroses and other bleeding episodes.
Newcombe et al. (1955)	12	35+	Normal to 65 mins	Abnormal	Haematuria haemarthroses, bruises.
Long et al (1955)	18	120–160	Normal	—	Menorrhagia dental extractions, *bruises*.
Bergsagel (1955b)	15	30	6½	21 /	Menorrhagia tonsillectomy dental extraction.
Biggs and Macfarlane* (1956) (a) (b)	12 17	18 42	Normal 9½	—	*Menorrhagia* Epistaxis, post-tonsillectomy
Quick, Pisciotta and Hussey (1955) (1) (2)	12 12	35–70 27–41	5–7 7–9	Normal Normal	Massive haematomata *cerebral haemorrhage* Menorrhagia dental extraction

* Not recorded elsewhere

and the correction of its abnormality by serum occurs quite frequently Studies of thromboplastin formation are rather incomplete at present but some information is now available

Dicoumarin anticoagulants

Biggs Douglas and Macfarlane (1953) showed that the serum from patients treated with the anticoagulant tromexan gave an abnormal thromboplastin generation Many observations have confirmed this finding and Hunter and Walker (1954b) and Verstraete and Vandenbrooke (1955) have found that the thromboplastin abnormality can be detected very early in treatment before Factor VII is much affected The abnormality in thromboplastin formation is corrected in some samples but not in others by addition of Christmas disease serum (which contains Factor VII) It is difficult to attribute the thromboplastin abnormality to lack of Factor VII Either the defect is due to Christmas factor deficiency or a new factor is involved as Koller (1954) suggests (see also Chapter VI)

Vitamin K Deficiency and Liver Disease and the Defect in New-born Infants

In the occasional cases of severe liver disease that we have seen the serum has given abnormal results in the thromboplastin generation test and the abnormality has not been corrected by the addition of serum from patients with Christmas disease These results suggest that the patients may also be deficient in the Christmas factor These findings are not at present reconcilable with those of Koller (see Chapter VI) but it is possible that different patients have different defects Bergsagel (1955) has studied one patient with severe obstructive jaundice and obtained similar results Van Creveld et al (1954) have found a similar serum abnormality in new-born infants

Congenital Factor VII Deficiency

Thirteen of the patients listed in Table 29 have been studied using the thromboplastin generation test The case of Jenkins (1954) has been re-investigated by Ackroyd (1956) and that of Owren (1952) has been re-studied by Owren Newcomb Hjort and Aas (1955) In all of these patients thromboplastin formation appears to be entirely normal The patients undoubtedly have Factor VII deficiency as judged by correction of the one-stage prothrombin time by normal serum and by an assay method involving Seitz filtered

able The relationship between one-stage clotting time and dilution of Factor VII is shown in Fig 36 using the plasma of patients treated with tromexan as a source of Factor VII deficient plasma. During the first few days of treatment with any of the dicoumarin group of drugs it is probable that Factor VII deficiency is the main cause of the lengthened one-stage prothrombin time From Fig 36 it will be seen that the method using undiluted plasma mixtures is insensitive to small reductions in Factor VII concentration Owren and Aas (1951) and Koller et al (1951) have therefore devised methods using greater dilutions A method has also been devised using Seitz filtered ox plasma (Owren and Aas 1951 Koller et al 1951) This method has the advantage that a supply of naturally occurring Factor VII deficient plasma is not necessary It has the disadvantage that the preparation of the Seitz filtered ox plasma may not be very easy

THE THROMBOPLASTIN GENERATION TEST AND DEFICIENCY OF FACTORS V AND VII

The results obtained with the thromboplastin generation test on patients with Vitamin K deficiency those treated with one of the dicoumarin group of drugs those with Factor V and Factor VII deficiency have been referred to in Chapter VI it is now proposed to summarize these results again more systematically

FACTOR V DEFICIENCY AND THROMBOPLASTIN FORMATION

So far the records of only two patients on whom the thromboplastin generation test has been carried out are known to us Bergsagel (1953) investigated a patient and found slight abnormality of thromboplastin formation using the patient s $Al(OH)_3$ treated plasma and normal platelets Since normal platelets are contaminated with Factor V much more definite abnormality might have been disclosed Horder (1955) and Horder and Sokal (1955) have also described a patient with abnormal thromboplastin formation in whom the Factor V deficiency was attributed to an ether soluble inhibitor The thromboplastin formation in Horder s case was quite markedly abnormal

FACTOR VII DEFICIENCY AND THROMBOPLASTIN FORMATION

Factor VII deficiency as judged by the one-stage prothrombin time

patient's plasma a belief which is supported by the fact that the one-stage prothrombin time of the patient's plasma was abnormal when Russell's viper venom and cephalin were used as thromboplastin (this thromboplastin being unaffected by Factor VII deficiency)

The patient's serum used in the thromboplastin generation test gave abnormal results complete correction was obtained by adding Christmas disease serum The thromboplastin generation defect was corrected by the alpha globulin fraction of normal serum but not the beta globulin fraction

To summarize the patient appears to be deficient in two substances Factor VII (beta globulin) and a second factor (alpha globulin) both factors influence the one-stage prothrombin time but the deficiency of the alpha globulin fraction influences the thromboplastin generation test and since it differs from Factor X (Koller 1955) is presumably a new factor The status of this factor is much the same as that of Factor X (see Chapter VI) The phenomena attributed to it have been established beyond reasonable doubt in mixture experiments on patients blood but there is no evidence about the mode of action of the factor in the normal clotting process The abnormality could well be referred to for identification as the Prower Defect (from the patient's surname) leaving the question of the presence or absence of a new factor for later experiments It is possible that the first two cases of de Vries et al (1955) are similar to Bergsagel's case In these patients the thromboplastin defect was corrected by Christmas disease serum

The case of Newcomb et al (1956) is also particularly interesting This patient usually had a grossly lengthened one-stage prothrombin time and on analysis the defect appeared to be identical with that caused by the dicoumarin drugs There was reduction in prothrombin and Factor VII and a serum defect in thromboplastin formation The one-stage prothrombin time was not corrected by additions of dicoumarin plasma The defect dates from early childhood and cannot possibly be attributed to any drug

The astonishing feature of this patient is the effect of fresh plasma transfusion Following 500 ml of plasma the patient's various clotting defects gradually disappear over a period of about 1 week. The relatively normal state is maintained for 3-4 weeks after which the tests revert to the previous abnormalities Bleeding manifestations are completely controlled by monthly plasma transfusions

ox plasma Factor VII was grossly diminished The clotting time was not corrected by plasma or serum from patients treated with the dicoumarol type of drugs These patients have been referred to in Chapter VI and the reactions of their blood to standard tests suggest that Factor VII may not be involved in the intrinsic blood thromboplastin system This belief is strengthened by the results of the clotting time prothrombin consumption test and thrombin generation test (Hicks 1955 Ackroyd 1955) which are normal

Other patients whose blood has been examined using the thromboplastin generation test are those of Stefanovic et al (1955) de Vries et al (1955) Newcomb et al (1955) Bergsagel (1955b) and Biggs and Macfarlane (1956) The five cases included by the work of Stefanovic et al de Vries et al are all characterized by a relatively short one-stage prothrombin time associated with a quite disproportionately severe bleeding tendency In general a one-stage prothrombin defect of the severity shown by these patients would be expected to cause no marked bleeding tendency Patients treated with dicoumarol anticoagulants are confidently maintained with one-stage clotting times exceeding those obtained in the blood of these patients All have quite abnormal thromboplastin formation if their sera are used to replace normal serum in the thromboplastin generation test Their defect is much more like that of Christmas disease than Factor VII deficiency and in one case the defect was not corrected by Christmas disease serum (de Vries (3)) No conclusions can be drawn about these patients except that they clearly differ quite markedly from the cases of Jenkins (1954), Hicks (1955) and Jurgens (1955)

Bergsagel (1955) and Telfer et al (1956) have studied one patient in great detail this patient was a female and had history of menorrhagia, bleeding after dental extractions and after tonsillectomy Her one-stage prothrombin time was prolonged and the Factor VII assay gave a value of 25 per cent Mixture of the patient s plasma with the plasma of patients treated with dindevan showed some mutual connection compatible with relatively high level of Factor VII in the patient s plasma Alpha and beta globulins obtained by electrophoresis of normal serum both had some ability to shorten the patient s one-stage prothrombin time Factor VII is associated with the beta globulin fraction (Owen and McKenzie 1954) These results suggest that deficiency of some factor other than Factor VII is concerned with the long one-stage prothrombin time of the

is the type caused by dicoumarol by Vitamin K deficiency, liver disease and seen in the congenital cases of Newcomb et al (1955) and possibly those recorded by us in Tables 29 and 30 These patients apparently have three deficiencies Factor VII prothrombin and a serum factor affecting thromboplastin formation which may or may not be the Christmas factor It is naturally tempting to suggest a common aetiology for these conditions the common link being Vitamin K the Vitamin K deficient cases being an uncomplicated lack of the factor the liver disease patients being unable to use the vitamin the dicoumarol drugs possibly interfering with the formation or action of Vitamin K and the congenital cases lacking some co-factor present in normal plasma which is essential for the proper use of Vitamin K. Naturally this is pure speculation but it is difficult not to try to associate these apparently diverse conditions which have a similar expression

The second group includes the four congenital cases of Jenkins (1954) Hicks (1955) Jurgens (1955) and Owren (1952) all of whom appear to have pure Factor VII deficiency which proves to be an unusually interesting condition because there is apparently no abnormality in the intrinsic blood coagulation mechanism Clotting is abnormal only in relation to tissue extracts

The third group includes the patients of Stefanovic et al (1955) and de Vries et al (1955) All have a relatively minor lengthening of the one-stage prothrombin time and a severe defect of thromboplastin formation The nature of the thromboplastin defect remains obscure in most of the patients As will be seen they are susceptible to no easy explanation Telfer s case (1956) is more definitely characterized because his and Bergsagel s study of the patient is more complete his case could easily be dignified by a newly named factor deficiency and further study may reveal that some of the less clearly defined cases are similar to his But in the present confused state it is probably wise to await the results of a more complete study of other patients and to be satisfied with the identifying name the Prower defect An unusual feature of all these patients is the normal prothrombin consumption test which most of the samples have shown This is easy to explain in the patients with pure Factor VII deficiency but less comprehensible in patients whose blood shows abnormal thromboplastin formation These findings are markedly different from those of the Factor V deficient patients who all have abnormal prothrombin consumption

Vitamin K_1 produced slight very temporary improvement of the patient's clotting defect The cases recorded by us in Table 29 may be similar to this case, some results obtained with the thromboplastin generation test are shown in Table 30 It will be seen that

TABLE 30

SOME RESULTS OF THE THROMBOPLASTIN GENERATION TEST CARRIED OUT ON THE BLOOD OF TWO PATIENTS WITH FACTOR VII DEFICIENCY

Source of $Al(OH)_3$ Plasma	*Source of Serum*	*Incubation time (Minutes)*				
		1	2	3	4	6
		Clotting time (Seconds)				
	Normal	45	9	7	7	8
	Patient (1)	60	50	25	13	14
	Patient (2)	77	60	54	44	20
	9 parts Patient (1) 1 part Christmas	19	10	10	10	10
	9 parts Patient (2) 1 part Christmas	56	15	12	12	12
	9 parts Patient (1) 1 part Dindevan	68	48	31	14	14
Normal	9 parts Patient (2) 1 part Dindevan	61	46	38	25	18
	9 parts Patient (1) 1 part Bergsagel s case	28	25	25	26	37
	9 parts Patient (2) 1 part Bergsagel s case	65	30	15	15	14
	9 parts Patient (1) 1 part Newcomb s case	77	55	45	26	15
	9 parts Patient (2) 1 part Newcomb s case	61	51	39	30	18

Christmas disease serum corrects the defect which is not corrected by serum from a patient treated by Dindevan or by serum from the patient of Newcomb et al Serum from Telford and Bergsagel s patient accelerates thromboplastin formation but does not correct the defect

It is difficult to draw any conclusions from these diverse patterns of results Careful analysis of these patients reveals a very complicated picture Two definite types can be selected from the patients so far studied The first and most commonly encountered

normal during two months as the patient recovered clinically This lack of correlation is also obvious in patients with cirrhosis of the liver (Table 31 and Donald et al 1954) Thus if a bleeding tendency exists or operation is contemplated it is wise to carry out both one- and two-stage tests before reaching any conclusions as to the cause of bleeding or the safety of operation

In haemorrhagic disease of the new-born also two-stage methods record low levels of prothrombin (Brinkhous Smith and Warner 1937)

PROTHROMBIN DEFICIENCY AND THERAPY WITH THE DICOUMARIN DRUGS

Usually the prothrombin is not greatly reduced by therapeutic levels of these drugs though it is possible that all may not have exactly the same effects In overdose of any of the drugs severe prothrombin deficiency may result and Donald et al (1954) have observed that the restoration of prothrombin to normal following Vitamin K treatment may be much slower than the reduction in one-stage prothrombin time

IDIOPATHIC OR CONGENITAL PROTHROMBIN DEFICIENCY

The majority of patients with hypoprothrombinaemia do not have true prothrombin deficiency as the main defect Of the congenital cases two patients recorded by Quick Pisciotta and Hussey (1955) (patients BB and VA) appear to come into this group though it is a pity that the diagnosis was not confirmed by any two-stage methods The one-stage prothrombin times of these two patients plasma samples were shortened by the addition of stored normal plasma but not by Factor V or Factor VII In the patient described by Hagan and Watson (1948) the one-stage clotting time was shortened by a preparation of prothrombin made by Seegers This purified prothrombin lacks Factor V but some samples may contain Factor VII The patients described by Ferguson (1950) did not lack Factor V but whether Factor VII or prothrombin were deficient was not discovered

Idiopathic Prothrombin Deficiency

Biggs and Douglas (1953) have reported a patient who acquired a coagulation defect in adult life This patient is of particular interest because he had uncomplicated prothrombin deficiency an abnor-

PROTHROMBIN DEFICIENCY

In a chapter on Hypoprothrombinaemia' it seems illogical to place prothrombin deficiency last but this deficiency in severe degree seems to be rare and relatively unimportant

VITAMIN K LIVER DISEASE AND PROTHROMBIN DEFICIENCY

The Vitamin K deficiency of obstructive jaundice steatorrhoea and infective hepatitis is associated with significant reduction in prothrombin Using a two-stage method Mann et al (1950) found quite a marked prothrombin reduction We have now studied a number of patients by the area method and quite marked prothrombin deficiency is common (Table 31) It will be seen that the low results

TABLE 31

ONE AND TWO STAGE PROTHROMBIN TESTS ON PATIENTS WITH VITAMIN K DEFICIENCY AND CIRRHOSIS OF THE LIVER

Condition	*One-stage prothrombin time in seconds*		*Results of the two-stage test in per cent prothrombin*
	Normal	*Patient*	
	12	14	36
Vitamin	13	15	54
K	15	18	48
Deficiency	12	19	28
	14	20	32
	16	23	37
	12	21	85
	12	23	73
	17	28	61
	15	29	32
	15	33	50
	12	33	48
	12	37	30
	18	120	15
	13	13	66
Cirrhosis	14	15	77
of the	12	14	14
Liver	17	18	36
	17	18	58
	14	17	83
	12	18	50

are not always associated with much alteration in the one-stage prothrombin time We followed one patient with infective hepatitis who had marked prothrombin deficiency the one-stage prothrombin time was restored almost to normal overnight following Vitamin K treatment but the two-stage test returned slowly to

normal during two months as the patient recovered clinically This lack of correlation is also obvious in patients with cirrhosis of the liver (Table 31 and Donald et al 1954) Thus if a bleeding tendency exists or operation is contemplated it is wise to carry out both one- and two-stage tests before reaching any conclusions as to the cause of bleeding or the safety of operation

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Idiopathic Prothrombin Deficiency

Biggs and Douglas (1953) have reported a patient who acquired a coagulation defect in adult life This patient is of particular interest because he had uncomplicated prothrombin deficiency an abnor-

mality which from the previous discussion is apparently very rare The case will be given in some detail

History The patient was a male aged 26 who had no bleeding tendency (although teeth and tonsils had been removed) until 15 months prior to his investigation Haemorrhagic episodes included haematuria epistaxis bleeding from the gums and large spontaneous bruises No obvious etiological factor could be found Clinical examination was negative

Preliminary Investigations

Haemoglobin	13 7 gm. per cent
White cell count	5100 per c mm
Plasma proteins	6 6 gm per cent
Albumin	3 8 gm per cent
Globulin	2 4 gm per cent
Fibrinogen	360 mg per cent

Liver Function Tests
- Thymol turbidity test negative
- Colloidal gold test negative
- Sucrose tolerance test normal
- Bilirubin 0 3 mg per cent

Fat excretion normal
- Intake of fat 280 gm.
- Total fat excreted 7 2 gm
- Per cent fat excreted 2 6

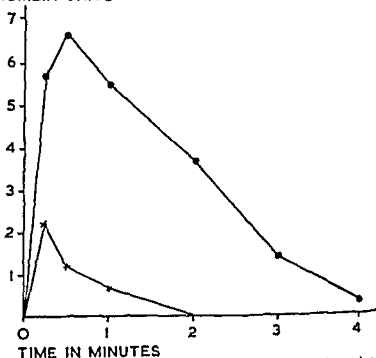


Fig 37 Thrombin formation tested by the two-stage method in normal plasma (●—●) and prothrombin deficient plasma (×—×)

Bleeding time 5 minutes (Ivy s method)
Tourniquet test negative
Platelets 645 000–1 121 000 per c mm

Invest gati n of Cl tt ng Defect

(1) Whole blood clotting time by the method of Lee and Wh te (1913)
13 20 minutes
(Normal 5 10 minutes)

(2) One-stage prothrombin time
( ) Normal 14 15 seconds. Patient 18 25 seconds

(3) Two-stage prothromb n test
By the two-stage method the amount of thrombin formed was greatly reduced Curves obtained with normal and the patient s plasma are shown in Fig 37 The amount of prothrombin assessed from the areas was 11 per cent of normal

(4) Factor V
The clotting time of the pat ent s plasma was not shortened by the addition of normal plasma adsorbed with $Al(OH)_3$ which adsorbs F ctor VII but not F ctor V Factor V was therefore not def cient.

(5) Factor VII
The amount of Factor VII was assessed by testing the ability of dilutions of the patient s plasma to shorten the clott g time of the plasma of a patient under treatment with tromexan. The patient s pla ma corrected the clotting defect in the tromexan plasma as well as did normal plasma. The o e-stage clotting time of the patient s plasma was not shortened by the presence of 10 per cent of normal serum There was therefore no deficiency of Factor VII

(6) Vitamin K
The p tient s clotting defect was not altered by 100 mg of synkavit g ven intravenously or by 500 mg of Vitam n K_1 orally or by 500 mg of Vitam n K_1 given intravenously The patient s clotting defect could not be attributed to Vitamin K deficiency

(7) No excess of inhibitory substances or abnormal ty of the thrombin-f brinogen reaction could be detected

The unusual features of this coagulation defect are the relatively slight change in the one-stage prothrombin time compared with the marked reduction in prothrombin recorded by the two-stage test and the lengthening of the whole blood clotting time which is possibly related to the slow generation of plasma thromboplastin when the normal amount of thrombin is not present The formation of thrombin in the patient s whole blood was much reduced (Fig 38)

Transfusion of this patient with normal plasma proved surprisingly ineffective in raising the level of prothrombin as measured by the area method On two occasions 500 ml of stored plasma and 850 ml of fresh plasma were transfused The levels of prothrombin before and after transfusion are shown in Fig 39 From these curves it will be seen that there was a very slight and transient rise in prothrombin Whether or not the rapid return of prothrombin to the pre-transfusion level indicates the usual rate of prothrombin utilization or whether or not the patient destroyed prothrombin unusually rapidly is not known

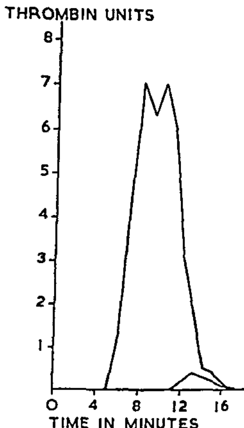


Fig 38 Thrombin formation in whol normal blood (upper curve) compared to thrombin formation in prothrombin deficient blood (lower curve) The test was carried out as described in Appendix III 9

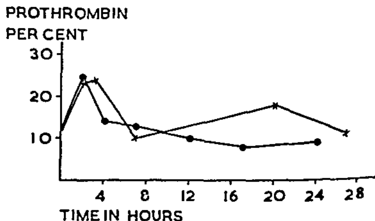


Fig 39 The results of the two-stage prothrombin test (area method) carried out on the plasma of the prothrombin deficient patient before and after transfusion with normal fresh plasma (●—●) and normal stored plasma (×—×)

MEASUREMENT OF PROTHROMBIN

Methods for the measurement of prothrombin in plasma have already been discussed in Chapter XI One-stage methods for the measurement of prothrombin with the exception of the method of Hjort Rapaport and Owren (1955) are unlikely to be successful The one-stage method using brain thromboplastin is too insensitive to changes in prothrombin Of the two-stage methods we think that the area method has advantages but if a method involving the destruction of antithrombin (Fantl 1954) gives a similar pattern of results then this method would probably be preferable

ABNORMALITY OF THE PROTHROMBIN TEST ASSOCIATED WITH THE PRESENCE OF INHIBITORY SUBSTANCES

The presence of heparin in the blood lengthens the one-stage prothrombin time and reduces the amount of thrombin detected by the two-stage method It is probably very seldom that the presence of heparin naturally circulating in the vessels causes a long one-stage prothrombin time In one patient described by Criselli and Cotellessa (1950) the presence of heparin in the blood caused a lengthening of the one-stage prothrombin time from a normal of 20 seconds to 25 seconds in a patient whose whole blood clotting time was 2 hours In patients who receive anticoagulant therapy with heparin the presence of heparin quite frequently causes a lengthening of the one-stage clotting time

Two further cases have been described in which a circulating anticoagulant which differed from heparin was present in the blood One of these patients described by Conley et al (1948) had chronic nephritis and had been treated for syphilis His whole blood had a clotting time of 60 minutes and the one-stage prothrombin time was 30 seconds where the normal clotting time was 16-20 seconds The patient s prothrombin-free plasma lengthened the prothrombin time of normal plasma The anticoagulant did not destroy thromboplastin and it is probable that this anticoagulant interfered with the reaction of prothrombin with thromboplastin A second case was described by Harrington et al (1950) and another by Frick (1955) The patient of Harrington et al was a female aged 60 with sciatica The whole blood clotting time was normal The one-stage prothrombin time was 50 seconds where the normal was 15

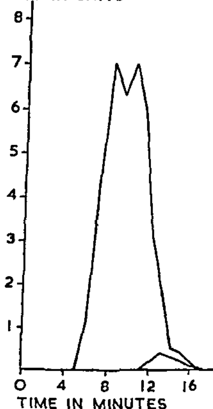


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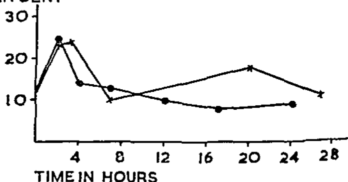


Fig 39 The results of the two-stage prothrombin test (area method) carried out on the plasma of the prothrombin deficient patient before and after transfusion with normal fresh plasma (●—●) and normal stored plasma (×—×)

on the origin of the defect In the congenital variety whether due to Factor V or Factor VII the most general feature is nose bleeding and two patients died of epistaxis Other common manifestations are bleeding from the gums spontaneous bruising menorrhagia and severe bleeding from injuries Haematuria is fairly common but is usually not the main complaint In four cases haemarthroses occurred (Rhoades and Fitzhugh 1941 de Marvel 1945 Frank 1950 Ferguson 1950) The occurrence of haemarthroses with this type of coagulation defect is interesting it is usually thought that haemarthroses are uncommon in haemorrhagic syndromes other than haemophilia It is possible that the long duration of the clotting defect is more important than its origin

In haemorrhagic disease of the new-born bleeding from the umbilical cord and gastro-intestinal tract are the most common In the Vitamin K deficiency associated with obstructive jaundice the main danger in the past has arisen from bleeding following surgical operations With Vitamin K therapy this seldom occurs Spontaneous bleeding is relatively uncommon in patients with obstructive jaundice when it does occur spontaneous bruising epistaxis haematuria and melaena are all described The patients with spontaneous Vitamin K deficiency for which no cause could be found suffered from epistaxis bleeding from the gums bruising menorrhagia haematuria abdominal bleeding and haemarthroses (Lewis and Bennett 1947 Ferguson 1950 Ley et al 1951)

In the dicoumarin poisoning which was first described in cattle large tissue haemorrhages and persistent bleeding after the operation of de-horning were recorded (Schofield 1924) In man the most characteristic and earliest sign of overdosage is haematuria As a rule if the administration of the drug is controlled by a reliable technique bleeding if it occurs is restricted to slight haematuria If a large overdose is given then extensive bleeding from other sites may occur

THE LABORATORY DIAGNOSIS OF HYPOPROTHROMBINAEMIA

A coagulation defect characterized by an abnormal reaction to tissue thromboplastin will be demonstrated by the one-stage method regardless of the cause of the defect The first step in the diagnosis of this type of coagulation defect therefore consists of carrying out

seconds The prothrombin-free plasma of the patient lengthened the prothrombin' time of normal plasma. The inhibitory action was apparently limited to human tissue thromboplastin. When rabbit, steer dog and sheep thromboplastins were used the normal and patient s plasmas had the same one-stage 'prothrombin time. The inhibitor was thermolabile and disappeared after 2 hours incubation at 37° C

A case with a similar species specific thromboplastin inhibitor was described by Fantl and Nance (1946) This patient differed from that of Harrington et al (1950) In Fantl and Nance s case the whole blood clotting time was prolonged and the abnormal reaction to thromboplastin could be demonstrated only with dilute thromboplastin, and the patient cannot therefore be said to have 'hypoprothrombinaemia

Favre-Gilly et al. (1951) described hypoprothrombinaemia' in patients with congenital heart disease In many of the patients the one-stage prothrombin time was grossly lengthened. Mixtures of the patient s and normal plasma had longer clotting times by the one-stage test than would have been expected by the author s method of assessing the results It is possible that the plasma of these patients contained some inhibitory substance

These cases are obviously very different in the mechanism of their coagulation defects It is possible that none of these patients had any reduction in prothrombin, Factor V or Factor VII in their plasma and yet in all the one-stage prothrombin time was lengthened.

FIBRINOGEN DEFICIENCY

A reduction in fibrinogen below about 100 mg per cent may cause a lengthening of the one-stage prothrombin time (Alexander de Vries and Goldstein 1949a) Dyggve (1947) records a patient with haemorrhagic scarlet fever in whose blood fibrinogen was reduced to very low levels and whose one-stage prothrombin time was greatly prolonged If the deficiency is entirely one of fibrinogen, then the two-stage method will give normal results and Factors V and VII will be found to be normal.

THE CLINICAL FEATURES OF 'HYPOPROTHROMBINAEMIA

The type of bleeding which occurs in the various syndromes associated with a prolonged one-stage prothrombin time depends

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THE CLINICAL FEATURES OF HYPOPROTHROMBINAEMIA'

The type of bleeding which occurs in the various syndromes associated with a prolonged one-stage prothrombin time depends

Absence of fibrinogen must always be suspected if the plasma fails to clot in the one-stage test Fibrinopenia can usually be detected in the one-stage test because a very flimsy clot forms In these cases the presence of a normal amount of prothrombin and Factor V may be demonstrated by appropriate tests

Patients with congenital or idiopathic Factor VII deficiency should also be studied using the thromboplastin generation test. A complete study of these patients using the maximum ingenuity is likely to be rewarding

THE TREATMENT OF HYPOPROTHROMBINAEMIA

There are two methods of treatment for haemorrhage associated with a prolonged one-stage clotting time The patient may be transfused with whole blood serum or plasma or Vitamin K may be administered.

Transfusion is surprisingly ineffective as a method of treatment except in the very rare congenital abnormality described by Newcomb et al (1955) In a patient with dicoumarin poisoning Croizat, Favre-Gilly and Milhet (1950) found that 1000 ml of fresh blood caused a slight fall in the prothrombin time which lasted only 24 hours Fresh plasma was no more effective In a similar case Owren (1950a) found that 500 ml of fresh serum gave a similar transient fall in clotting time In the case of Factor V deficiency described by Owren (1947) Factor V isolated from 200 ml of fresh plasma gave a rise to about 5 per cent of Factor V and after three days the effect of the injection had disappeared The patient with Factor VII deficiency described by Alexander et al (1951) was a child aged four The administration of 500 ml of fresh blood to this patient caused a fall in the one-stage clotting time from 72 to 55 seconds The patient with prothrombin deficiency described earlier in this chapter was given 850 ml of fresh plasma and the rise in prothrombin was slight and transient In the patient with congenital heart disease described by Favre-Gilly et al (1951) the effect of transfusion was more sustained but it was necessary to give 3000 ml. of plasma before operation was undertaken Thus if transfusion of blood, plasma or serum is used as a treatment the volume given must be large and the *transfusions repeated daily*

Vitamin K is an effective form of treatment in patients with obstructive jaundice steatorrhoea or haemorrhagic disease of the

the one-stage test If the one-stage test gives abnormal results then some indication of the probable abnormality may be obtained from the clinical features of the case If the patient is an infant less than ten days old or if the patient has jaundice or steatorrhoea there may be lack of Vitamin K Vitamin K may then be administered and a return of the prothrombin time to normal within twenty-four hours confirms the diagnosis If a water soluble Vitamin K analogue is ineffective then Vitamin K_1 should be given

In patients with no jaundice who are more than two weeks old and whose one-stage prothrombin time is not restored to normal by Vitamin K_1 further tests are required The first step is to test the reaction of plasma to thrombin and to measure the antithrombin content of the plasma Abnormality in these tests may, rarely, account for a lengthening of the one-stage clotting time and unless these tests are normal the two-stage prothrombin test cannot measure prothrombin

The qualitative tests for the distinction between prothrombin Factor V and Factor VII deficiency should then be made Factor V deficiency can be detected by adding 10 per cent of $Al(OH)_3$ treated plasma If Factor V is deficient then the one-stage clotting time will be shortened The addition of dicoumarin or tromexan plasma will also shorten the clotting time of the patient s plasma If $Al(OH)_3$ treated plasma does not shorten the clotting time Factor V is not lacking and 10 per cent of normal serum should be added If serum shortens the one-stage clotting time Factor VII is deficient and the clotting time will not be shortened by the addition of tromexan plasma If Factors V and VII are not deficient then 10 per cent of normal plasma is added If this addition shortens the clotting time then probably prothrombin is reduced If the addition of normal plasma is ineffective the presence of an inhibitor should be suspected

If the defect appears to be an isolated reduction in either Factor V or Factor VII then the extent of the abnormality can be assessed quantitatively by the one-stage test If prothrombin is reduced the defect can be measured by the two-stage test Combined deficiencies are distressingly common

The presence of an inhibitor may be suspected if a mixture of equal parts of the patient s and normal plasma has a one-stage clotting time of more than two seconds longer than the normal plasma If heparin is present the inhibitory action is removed by the addition of toluidine blue (Appendix IV 23 and 26)

Absence of fibrinogen must always be suspected if the plasma fails to clot in the one-stage test Fibrinopenia can usually be detected in the one-stage test because a very flimsy clot forms In these cases the presence of a normal amount of prothrombin and Factor V may be demonstrated by appropriate tests

Patients with congenital or idiopathic Factor VII deficiency should also be studied using the thromboplastin generation test. A complete study of these patients using the maximum ingenuity is likely to be rewarding

THE TREATMENT OF HYPOPROTHROMBINAEMIA'

There are two methods of treatment for haemorrhage associated with a prolonged one-stage clotting time. The patient may be transfused with whole blood serum or plasma, or Vitamin K may be administered.

Transfusion is surprisingly ineffective as a method of treatment except in the very rare congenital abnormality described by Newcomb et al (1955) In a patient with dicoumarin poisoning Croizat, Favre-Gilly and Milhet (1950) found that 1000 ml of fresh blood caused a slight fall in the prothrombin time which lasted only 24 hours Fresh plasma was no more effective In a similar case Owren (1950a) found that 500 ml. of fresh serum gave a similar transient fall in clotting time In the case of Factor V deficiency described by Owren (1947) Factor V isolated from 200 ml of fresh plasma gave a rise to about 5 per cent of Factor V and after three days the effect of the injection had disappeared The patient with Factor VII deficiency described by Alexander et al (1951) was a child aged four The administration of 500 ml of fresh blood to this patient caused a fall in the one-stage clotting time from 72 to 55 seconds The patient with prothrombin deficiency described earlier in this chapter was given 850 ml of fresh plasma and the rise in prothrombin was slight and transient. In the patient with congenital heart disease described by Favre-Gilly et al (1951) the effect of transfusion was more sustained but it was necessary to give 3000 ml. of plasma before operation was undertaken Thus if transfusion of blood, plasma or serum is used as a treatment the volume given must be large and the transfusions repeated daily

Vitamin K is an effective form of treatment in patients with obstructive jaundice steatorrhoea or haemorrhagic disease of the

new-born The fall in the one-stage clotting time after the administration of Vitamin K usually occurs within twenty-four hours and the one-stage test can be maintained at a normal level by repeated doses of Vitamin K If the patient is bleeding at the beginning of treatment Croizat et al (1950) suggest that a transfusion should be given with the first dose of Vitamin K In these patients the primary abnormality appears to be a deficiency of Vitamin K Both the water soluble analogue and Vitamin K_1 are effective though Quick and Collentine (1951) have shown that Vitamin K_1 has a smaller therapeutic dose Before operation on patients with jaundice the one-stage test should always be carried out and Vitamin K administered before and after operation In dicoumarin and tromexan therapy the water soluble *Vitamin K may have no effect but Vitamin K_1 is more generally* reliable There is as yet no information as to which of the coagulation factors is most influenced by Vitamin K It seems probable that both prothrombin and Factor VII are involved

SUMMARY TO CHAPTER XIV

The publication of Quick's one-stage prothrombin time test in 1935 was followed by extensive application of the test to the study of liver disease and Vitamin K deficiency to the study of spoiled sweet clover disease in cattle and to the application of the haemorrhagic agent dicoumarin to the treatment of thrombosis The value of this work was in no way affected by the contemporary assumption that all the conditions studied were due to prothrombin deficiency

Later work has shown that true prothrombin deficiency is rare but that two other factors Factors V and VII also affect the one-stage prothrombin time and that one or other of these factors is deficient in most patients whose plasma has a long one-stage prothrombin time

The use of the thromboplastin generation test on the blood of patients with Factor VII deficiency has revealed yet further complications because it appears that several different defects may be included within this general category

CHAPTER XV

HAEMOPHILIA CHRISTMAS DISEASE AND RELATED CONDITIONS

During the past few years important changes have taken place in the concept of haemophilia Previously haemophilia was regarded as a well defined clinical entity with a characteristic symptomatology and inheritance caused by a specific defect of coagulation Recent advances in knowledge of the thromboplastin-forming system of the blood have altered these views and it seems now that the term haemophilia as used in the past, covered a group of conditions in which similar clinical manifestations were produced by different basic defects in the coagulation mechanism The presentation of these changed ideas can best be achieved by a brief review of their evolution

HISTORICAL

Early in the last century the occurrence of constitutional conditions marked by excessive bleeding from slight injuries began to be recognized including a striking haemorrhagic state which affected males and was inherited by them through apparently normal females Many names were applied to these conditions among them the term haemophilia which was introduced by Hopft in 1828 Later in the century there was an increasing tendency to restrict the name haemophilia to cases of the inherited haemorrhagic disease of males but it was often applied to conditions now recognizable as von Willebrand s disease telangietasia, thrombocytopenic purpura and even scurvy and cases of local bleeding due to organic disease The final establishment of haemophilia as a specific clinical entity was made by Bullock and Fildes (1911) in their monumental survey of the whole of the relevant literature then available As a result of a study of their data, it became accepted by most authorities that the term should only be used if certain diagnostic criteria were satisfied These included the liability to excessive bleeding which had existed from infancy and was restricted to the male sex evidence of similarly affected males in the family and of transmission only by

the apparently normal female the demonstration of a prolonged blood clotting time and the absence of any other abnormality that might cause bleeding These restrictions produced a great clarification of a previously confused group of haemorrhagic conditions and for many years formed the basis for the careful diagnosis of haemophilia Increasing experience while it confirmed the major diagnostic points has necessitated some important modifications It was soon recognized that the inheritance pattern of haemophilia indicates that the defective gene is located on the X chromosome Theoretically therefore the daughters of a haemophilic male must receive the abnormal gene from him and are thus all carriers of haemophilia and a female should suffer from active haemophilia if her mother was a carrier and her father a haemophilic and she received an abnormal gene from each parent These theoretical considerations which ran counter to the classical view that males cannot transmit haemophilia and females cannot suffer from it have now been proved correct by abundant evidence from human haemophilic families and from breeding experiments in haemophilic dogs It was also found that there is probably a high mutation rate in haemophilia since many instances exist in which the abnormal gene has appeared in the male or female without previous family history

It was the study of the blood coagulation defect in haemophilia which has produced the most radical changes Following the demonstration by Wright in 1893 of the prolonged clotting time of the blood in haemophilia it was established that some disorder of coagulation was probably the cause of the haemorrhagic symptoms but the nature of this defect eluded identification for many years despite the efforts of almost every worker engaged in blood coagulation research All the factors of the classical theory of coagulation were in turn thought to be deficient in haemophilia and were subsequently shown to be normal yet it was known from the experiments of Addis (1911) and Govaerts and Gratia (1931) that the addition of a small proportion of normal plasma to haemophilic blood corrected its delayed coagulation time It was not until similar experiments had been carried out in 1937 by Patek and Stetson that there was a general acceptance of the idea that haemophilic blood lacks a factor present in normal plasma but not identifiable with any of the factors of the classical theory of coagulation This factor called antihaemophilic globulin by Patek and Taylor (1937)

could not be fitted into existing theory because there were no means by which its function in normal coagulation could be demonstrated Even its presence or absence in a given preparation could only be assumed from the effect of adding the preparation to haemophilic blood since normal blood clotting systems could not be deprived of antihaemophilic activity and thus could not function as indicators Thus a dangerously circular definition of both the clotting factor and of haemophilia established itself haemophilia is an inherited deficiency of antihaemophilic globulin antihaemophilic globulin is the factor which is deficient in haemophilia In such a situation the clinician relied upon the pathologist to confirm a diagnosis of haemophilia by demonstrating the lack of antihaemophilic globulin which the pathologist could only do by relying upon a previous clinical diagnosis of haemophilia Despite these obvious fallacies attempts to measure the deficiency of antihaemophilic globulin in cases of different grades of severity gave useful information This work was assisted by other tests of clotting function which though less specific than estimates of antihaemophilic globulin, were more sensitive indicators of the haemophilic defect than simple determinations of clotting time The most useful of these was the prothrombin consumption test introduced by Quick (1943) which measured the amount of prothrombin remaining in the serum one hour after coagulation was complete In normal blood very little prothrombin can be found in the serum but in haemophilia the serum has great prothrombin activity Using this test and also assays of antihaemophilic globulin Merskey (1949) was able to show that there existed a number of families suffering from a relatively mild form of haemophilia in which the clotting time might be within normal limits but prothrombin consumption and A H G levels grossly abnormal Such patients though clinically less severely affected than those with high grade haemophilia were still liable to bleed dangerously from slight injuries or operation sites

Increased interest in cases on the border line of typical haemophilia led to studies of possible A H G deficiency in atypical cases Usually the blood from the unproved case was mixed with blood from a known haemophilic in order to observe the degree of correction of the known haemophilic clotting defect by the 'unknown blood If there was good correction then the test blood sample was assumed to have A H G activity and the diagnosis of haemophilia was refuted if there was no correction then A H G activity was absent

and the diagnosis of haemophilia, if not absolutely confirmed was at least very probable Such an argument obviously relied on the accuracy of the diagnosis of the haemophilia in the donor of the standard blood and this diagnosis in the absence of any independent method for detecting A H G deficiency, relied almost entirely on clinical manifestations The inherent danger of this method became obvious when it was found by several workers (Pavlovsky 1947 Shulman and Smith 1952 Poole 1953) that blood samples from cases clinically resembling haemophilia might mutually correct each other suggesting that different basic defects might exist within the clinical definition The development of the thromboplastin generation test allowed a clarification of this situation by providing a method for detecting A H G activity that did not rely on haemophilic blood as an indicator Biggs et al (1953) found seven cases of supposed haemophilia in which there was no deficiency of A H G in the blood but a deficiency of another factor not previously recognized which is normally required for thromboplastin generation This factor differed from A H G in being present in normal and haemophilic serum as well as plasma and in being adsorbed by $Al(OH)_3$ and in not being consumed during coagulation It was called Christmas factor from the surname of the first patient investigated and the haemorrhagic condition was called Christmas disease Simultaneously and independently Aggeler et al (1953) in the United States investigated a case clinically resembling haemophilia in which the clotting defect was due to the deficiency of a previously unrecognized factor concerned with plasma thromboplastin This factor was shown to be present in serum adsorbed by barium sulphate and not consumed during clotting These workers called this factor P T C (plasma thromboplastin component) and the clinical state caused by its deficiency P T C deficiency There is now little doubt that P T C and Christmas factor are identical Following these publications many reports of cases identified as Christmas disease have appeared as in two of the cases published by Biggs et al (1953) showing evidence of a sex linked inheritance like that of true haemophilia The incidence of this condition relative to haemophilia seems to vary widely from one country to another being about 1 to 10 in England and 1 to 3 in Switzerland With the recognition of Christmas disease certain anomalies of the past have been cleared away Instances have been discovered in which known haemophilic subjects whose blood was used to test the diagnosis in

other patients or for experimental work on coagulation were actually suffering from Christmas disease Cases of supposed haemophilia in which the injection of serum was effective or the administration of A H G preparation was ineffective may have been cases of Christmas disease Many famous haemophilic families including that of the Tenna valley in Switzerland are now identified as suffering from Christmas disease

Following the separation of Christmas disease from true haemophilia, evidence has been produced that other distinct conditions may exist within the same group Rosenthal et al (1953) have described cases resembling haemophilia in which neither A H G nor Christmas factor are deficient and in which the clotting defect has been ascribed to deficiency of a third factor supposedly required for thromboplastin formation This has been called P T A (plasma thromboplastin antecedent) but the actual existence of this factor and of the clinical state thought to be due to its deficiency seems not to have been clearly established and we therefore refer to it as Rosenthal s syndrome

It has also been recognized that a deficiency of factors required for thromboplastin formation may occur as acquired or secondary conditions In most instances the apparent deficiency is due to the development of a circulating coagulant which causes defective thromboplastin formation These anticoagulants may arise during or following pregnancy or after repeated transfusion and may thus be of the antibody type Occasionally as in the case described by Joules and Macfarlane (1938) an idiopathic deficiency of A H.G may develop in an adult patient Deficiency of Factor V or Factor VII causes haemorrhagic states that are distinguished from the haemophilia-like conditions by the occurrence of a prolonged one-stage prothrombin time and they are dealt with in Chapter XIV

Not unnaturally there has been considerable controversy concerning the nomenclature of these haemophilia-like conditions Reasons for and against the use of the name Christmas disease have been discussed in the literature and need not be enlarged upon here Many alternative suggestions have been made Aggeler et al (1954) abandoned the name P T C deficiency in favour of plasma-thromboplastin Factor B deficiency or deuterohaemophilia Graham and Brinkhous (1953) retained haemophilia for A H G deficiency and referred to other similar conditions as haemophiloid states designated by the letters of the alphabet Christmas disease

being haemophiloid state C, and P T A deficiency being haemophiloid state D Wiener (1953) has suggested Haemophilia I for haemophilia and 'Haemophilia II for Christmas disease Koller (1954) refers to Factor VIII deficiency' and Factor IX deficiency In Europe A H G deficiency and Christmas disease are being increasingly referred to as 'Haemophilia A' and 'Haemophilia B respectively a nomenclature that Pitney and Dacie (1955) feel will prove most widely acceptable

HAEMOPHILIA (HAEMOPHILIA A)

Clinical Manifestations Except in very mild cases the haemorrhagic tendency appears in infancy and remains a life-long affliction Bleeding may occur from or into the external surfaces of the body, from the nose mouth gastro-intestinal or renal tracts and may continue for days or weeks leading in some cases to death from exsanguination There may be haemorrhage into the tissues following trauma or infection resulting often in the formation of enormous haematomas In addition to the blood loss these tissue haemorrhages may cause serious pressure effects The blood supply to limbs or abdominal organs may be occluded with subsequent necrosis or atrophy and bleeding into the tissues of the mouth or neck has caused many cases of fatal asphyxia

A feature thought to be characteristic of haemophilia but now known to occur in other conditions associated with clotting defects of long duration is the occurrence of repeated haemorrhages into the joints These haemorrhages cause great pain and swelling and after each of the often repeated episodes joint function is progressively impaired and serious crippling is usual

In any one individual the severity of the basic disorder tends to remain relatively constant There may be periodic phases during which the patient seems to be rather more or less liable than usual to develop haemorrhages but such phases are very indefinite and cannot be correlated with demonstrable changes in the clotting mechanism In some cases in which middle life has been reached there appears to be a lessening of the tendency to bleed

There are very wide variations in severity from one patient to another though in any one family the different members are usually affected to the same degree Patients and families can be graded as regards the severity of their defect on clinical or laboratory findings

and the two sources of information usually agree reasonably well On clinical grounds a patient would be graded as severe if he suffered repeated haemarthroses with serious crippling and deep tissue haemorrhages with little provocation as moderate if he had few haemarthroses and no serious crippling and an occasional haematoma and mild if he had only an occasional haemarthrosis or none and merely gave a history of abnormal bleeding after definite injury

Incidence In the absence of adequate diagnostic facilities in many parts of the world the incidence of haemophilia in different races cannot be assessed The greatest number of reported cases occurred among the Germanic peoples of Northern Europe Great Britain and North America In this country the condition appears to affect about 2-3 persons per 100 000 of the population

Fewer cases are reported among the Latin races and in the Asiatic and Negro peoples it seems to be almost unknown (Nesbitt and Richmond 1949)

It is now established that a condition genetically and pathologically indistinguishable from haemophilia occurs in dogs (Hutt, Rickard and Field 1948 Graham Buckwater Hartley and Brinkhous 1949) From an experimental point of view it is unfortunate that this canine condition is extremely rare Though haemorrhagic states affecting other animals may present a clinical picture resembling haemophilia the basic defects appear to be different, as for example in the case of the so-called haemophilia of pigs (Hagan Muhrer and Bogart 1941)

Heredity One of the most striking aspects of haemophilia, which it shares with Christmas disease is its mode of inheritance and its almost exclusive limitation to the male sex It now appears to be reasonably certain that the defect is carried by the X-chromosome and that it is inherited as a sex-linked recessive character (Fig 40) The condition is thus transmitted to affected males by their mothers who themselves have no detectable haemorrhagic tendency resembling haemophilia though there may occasionally be abnormal bleeding of another type (Merskey and Macfarlane 1951) The male haemophilic does not pass his defect to his sons but his daughters must be potential transmitters of the condition The children of a female transmitter have an equal chance of being normal or of being transmitters if girls or haemophilics if boys Since the female transmitters are almost invariably heterozygous for the condition they do not exhibit any abnormality In the extremely rare event of a

marriage between a haemophilic male and a female transmitter homozygous females might be produced and these would be expected to exhibit classical haemophilia It is almost certain that the haemophilic females described by Merskey (1951b) and Israels Lempert and Gilbertson (1951) and Pinniger and Franks (1951) are

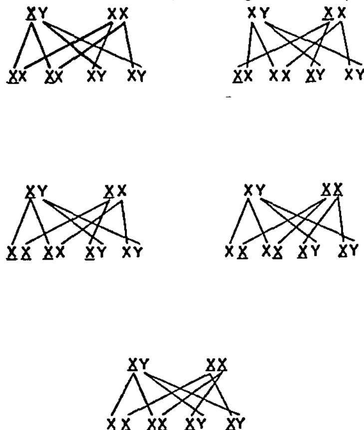


Fig 40 The inheritance of haemophilia. The X chromosome carrying the haemophilic defect is marked X Female carriers of the disease are of constitution XX female haemophilics XX and male haemophilics XY

authentic examples of this type of inheritance The many other reported cases of haemophilia in women have little claim to this diagnosis and have been reviewed by Merskey (1951b) and Israels et al (1951) In haemophilic dogs it has been shown that the offspring of matings between affected males and carrier females include the expected proportion of females with active haemophilia (Brinkhous and Graham 1950) In a number of instances an acquired clotting

defect in women closely resembling that of true haemophilia has been reported and this condition will be discussed in Chapter XVII

True haemophilia occurs sporadically in children without previous family history, in about 25-30 per cent of the total cases recorded There is no reason to believe that the sporadic condition differs in any way from the hereditary state already described It is probably due to a mutation so that either haemophilic males or female transmitters may arise apparently de novo The observation by Quick and Conway (1949) that haemophilia occurred in one of a pair of identical twins is of considerable genetic interest

HAEMOPHILIA AS A SOCIAL PROBLEM

Despite its comparative rarity haemophilia is for important reasons a social problem of considerable magnitude The expectation of life has been assessed at about sixteen years (Andreassen 1943) but many haemophilics live for considerably longer than this Throughout the whole of their lives these patients exist on the brink of disaster and most of them spend a very considerable proportion of their lives in hospital The incessant anxiety suffered by the parents of haemophilic children must also be taken into account, and the education of children who must be treated with extreme care and who are frequently crippled and incapacitated for long periods is a problem of the greatest difficulty The plight of the girls in haemophilic families though less appreciated is also deplorable Since there is as yet no reliable way of recognizing the female carrier of the haemophilic gene it cannot be known if any particular girl in a haemophilic family is liable to transmit the condition to her children or not an uncertainty that has obvious and serious emotional consequences

THE CLOTTING DEFECT IN HAEMOPHILIA

The Nature of the Haemostatic Defect

Available evidence suggests that the haemostatic defect in haemophilia is due only to the inefficient coagulation Liston (1839) seems to have been the first to recognize that the slow coagulation of haemophilic blood was the cause of the clinical condition observing that there seemed to be a want of coagulability and deficient fibrine Later authorities thought that weakness of the blood vessels was the major factor (Legg 1872 Moreton 1886) or that

marriage between a haemophilic male and a female transmitter homozygous females might be produced and these would be expected to exhibit classical haemophilia It is almost certain that the haemophilic females described by Merskey (1951b) and Israels, Lempert and Gilbertson (1951) and Pinniger and Franks (1951) are

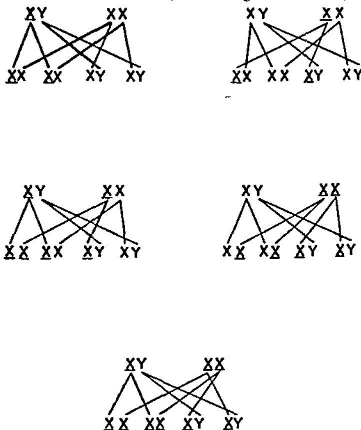


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mality probably an increased stability (Howell and Cekada 1926 Fonio 1932 Birch 1932) On the other hand Feissley (1924) showed that both normal and haemophilic platelets were equally active in hastening the clotting of normal blood and supposed that some normal plasma factor was concerned with platelet action Govaerts and Gratia (1931) provided evidence in favour of this hypothesis and considered that the effectiveness of normal platelets to clot haemophilic blood was due to a normal plasma factor associated with them which was lacking in haemophilic plasma Finally, Patek and Stetson (1936) showed that if normal platelets were carefully washed free of plasma they were no more effective than haemophilic platelets in clotting haemophilic blood

The attempts to explain the apparent 'thromboplastin deficiency' of haemophilic blood on the basis of a platelet defect has thus led research back to the plasma Addis (1911) had provided the key to the problem when he demonstrated that the addition of a small proportion of normal plasma, or of a globulin fraction derived from it to haemophilic blood shortened its clotting time to normal Unfortunately Addis considered that the only clotting factor present in this globulin fraction was prothrombin and concluded that there was a prothrombin deficiency in haemophilia This conclusion was subsequently shown to be wrong and for some reason his experiments were not repeated until 1936 when Patek and Stetson, by an almost identical technique demonstrated the corrective effect of adding to haemophilic blood a small amount of globulin material derived from normal plasma This time there was no attempt to explain its effect on the basis of a known clotting factor it became accepted that the normal plasma contained a previously unrecognized factor antihaemophilic globulin which was shown to be absent from haemophilic blood (Minot and Taylor 1947)

The first indications of the probable physiological role of antihaemophilic globulin and thus of the nature of the defect in haemophilia came from the experiments of Quick (1947) and Brinkhous (1947) These provided a link between A H G and the platelets both of which were shown to be required for normal coagulation after contact of the plasma with glass Quick (1947) suggested that A H G was the precursor of thromboplastin and was activated by the platelets he therefore renamed A H G thromboplastinogen' and haemophilia hypothromboplastinogenaemia Brinkhous (1947) considered that the A H G lysed the platelets being a throm-

haemorrhage was simply due to an increased blood volume (Immermann 1879) With the introduction of a method for determining the coagulation time by Wright (1893) the importance of the clotting defect became generally recognized and Addis (1910) concluded that it was the only cause of the haemorrhagic state Pavlovsky (1950) has revived the old suggestion that an undue fragility of the blood vessels plays some part in producing the haemorrhagic condition

Though there is little evidence to substantiate the view that there may be a vascular factor in haemophilia it has always been difficult to explain solely on the basis of the coagulation defect, the apparently spontaneous haemorrhages that may occur into the joints and from the kidneys in many cases and the occurrence of periods of increased liability to bleed which cannot be related to demonstrable changes in clotting function

The Nature of the Clotting Defect

Until 1947 understanding of the nature of the clotting defect in haemophilia was hampered by the acceptance of the classical theory of coagulation which formed too rigid a basis for most experimental work The classical theory could provide no explanation for the slow clotting of haemophilic blood since all the factors recognized were successively shown to be quantitatively and apparently functionally normal in haemophilia The demonstration by Quick Stanley-Brown and Bancroft (1935) that the one-stage prothrombin time test gave normal results with haemophilic blood established the fact that its ability to react to tissue thromboplastin was normal and suggested that there was some fault in the thromboplastic system in haemophilia But the classical theory contemplated 'thromboplastin only in terms of tissue extracts and possibly, of the platelets It was known that the thromboplastic activity of haemophilic tissues was normal when tested on normal blood (Minot and Lee 1916) or on haemophilic blood (Brown 1952) and the platelets thus received some short-lived attention

Minot and Lee (1916) stimulated the idea that the platelets were in some way defective in haemophilia when they showed that the addition of normal platelets to haemophilic blood corrected the clotting defect The failure of the platelets in haemophilic blood to rupture on contact with a foreign surface as do the platelets in normal blood, seemed to confirm the existence of a platelet abnor-

number of cases of haemophilia and other coagulation defects leave little doubt that A H G deficiency is an essential cause of the haemophilic clotting defect The severity of the haemorrhagic symptoms is well correlated with the A H G concentration in the blood in most cases and we have found that the haemostatic mechanism can be restored to normal if the blood level of A H G is artificially raised by an intravenous injection of a potent A H G preparation

Though it is possible that a simple deficiency of A H G itself the result of a genetic defect may be responsible for all the manifestations of haemophilia there are indications that other complications lie behind this deficiency The symptoms of diabetes and pernicious anaemia are due to a deficiency of insulin and Vitamin B12 and are alleviated by the administration of these substances but the mechanism by which these deficiencies arise are still obscure Similarly the origin of A H G deficiency may involve other coagulation factors or inhibitors in ways not yet recognized Tocantins (1942 1943 1944 1945) has provided much evidence to support his view that there is an excess of an inhibitor in haemophilia if such an inhibitor inactivated A H G or prevented the reactions in which it takes part the result would be an apparent deficiency of A H G There are further indications that factors other than A H G deficiency may play a part in haemophilia There seems to be less correlation between A H G blood levels and the results of the prothrombin consumption test than would be expected if A H G was the only variable factor influencing clotting efficiency the amounts of A H G which need to be given in vivo in order to produce a therapeutic effect greatly exceed the amount calculated from in vitro results an apparent deficiency of A H G may occur in von Willebrand s disease but the haemorrhagic symptoms in this condition are quite different from and much milder than those which occur in haemophilia It is possible that these apparent anomalies arise from unsuspected technical complications inherent in the method of assaying A H G activity and in other tests of clotting function but for the present an open mind must be kept regarding the causes of the abnormal bleeding in true haemophilia

THE LABORATORY FINDINGS IN HAEMOPHILIA

In a case of severe haemophilia the findings are usually definite and characteristic but in mild or atypical cases considerable care may be

bocytolysin and that the platelets then released thromboplastin The important idea common to both these interpretations was that A H G and the platelets react in some way to generate thromboplastin in the blood and that a deficiency of A H G causes defective coagulation because the generation of blood thromboplastin is deficient This idea was confirmed and extended by the experiments of Biggs Douglas and Macfarlane (1953a b) in which the existence of a complex thromboplastin-forming system was demonstrated involving not only A H G and the platelets but also Christmas factor and Factor V The recent work of Bergsagel (1955) supports the idea that A H G is concerned with the lysis of the platelets He has provided evidence that following contact with a foreign surface A H G Christmas factor and calcium react to form an intermediate product and that this intermediate product then causes the platelets to undergo the viscous metamorphosis described by Wright and Minot (1917) As the result of these changes a granular material is discharged from the platelets which in the presence of Factor V is extremely active in converting prothrombin to thrombin A deficiency of A H G will prolong the time which is required for these initial changes to take place and thus will prolong the clotting time of the blood perhaps more important it will reduce the amount of thromboplastin formed and the amount of prothrombin which is converted during the process of coagulation

The introduction of a method for assaying antihaemophilic globulin which is independent of a supply of haemophilic blood (Biggs Eveling and Richards 1955) has facilitated the study of the incidence and effects of A H G deficiency In previous methods (discussed by Alexander and Landwehr 1948b) the A H G activity of an unknown sample was judged from its ability to correct the clotting time or prothrombin consumption of haemophilic blood or plasma and they have the practical disadvantage that haemophilic blood is not always available when required The method of Biggs et al (1955) is based on the thromboplastin generation test and measures the amount of thromboplastin generated in a system in which A H G concentration is the controlling factor It is sensitive to 50 per cent changes in A H G concentration and the results can be expressed in terms of an arbitrary unit (see Appendix IV 30) or as a percentage of the average normal plasma concentration

The results of the application of this assay method to a large

probably indicates a severe defect a short clotting time does not necessarily mean that the patient is mildly affected and has little diagnostic or prognostic significance Clotting times carried out in silicone coated tubes and the clotting time of recalcified blood or plasma or of plasma after removal of platelets by centrifuging have all been recommended as methods for demonstrating the haemophilic defect They appear to have little more value than the simple Lee and White clotting time determination

PROTHROMBIN CONSUMPTION TEST

Brinkhous (1939) showed that after the coagulation of haemophilic blood was apparently complete the serum still contained a large amount of unconsumed prothrombin which can be activated to thrombin by the addition of tissue thromboplastin This observation has been used as the basis for a practical test of clotting efficiency by Chevallier et al (1946) Quick (1947a) Soulier (1948a b) and Merskey (1950b) Though not specific for haemophilia this test is a more sensitive indicator of deficient thromboplastin formation than is the simple clotting time and it is useful in the diagnosis and assessment of the severity of the defects of this type

Normal serum examined one hour after coagulation contains little prothrombin using the prothrombin consumption index (Merskey 1950) values of 20 per cent are not exceeded In haemophilia and related conditions the activity of the serum exceeds this value and in severe cases may be apparently greater than 100 per cent indicating an apparent increase in prothrombin during clotting These surprisingly high values are probably due to the development in the serum of factors which potentiate the action of the tissue thromboplastin used to convert the prothrombin (Alexander and de Vries 1949a Langdell Graham and Brinkhous 1950) The test considerably increased the precision of quantitative investigations in haemophilia because even in cases in which the clotting time was normal prothrombin consumption was often grossly abnormal (Merskey 1951a) and it could be used also as a basis for judging the corrective effect on haemophilic blood of the addition of plasma or plasma preparations and thus for roughly assaying their A H G content Prothrombin consumption is also influenced by a deficiency of any of the factors involved in thromboplastin generation including the platelets (Buckwalter Blythe and Brinkhous 1949 Merskey 1950a)

necessary to establish the nature of the defect The platelet count, tourniquet test and bleeding time are usually normal, but an occasional prolongation of the bleeding time, as measured by the Ivy technique, has been observed in one or two cases of otherwise typical haemophilia Clot retraction is difficult to measure because of the slow and incomplete coagulation of haemophilic blood but it appears to be normal The fibrinolytic system also appears to be normal haemophilic clots being as stable as normal clots and activation by *streptokinase* or *chloroform in vitro* or by adrenalin in vivo follows a normal pattern

The extent of the delay in the clotting time as measured by the Lee and White method varies a great deal from one patient to another but tends to remain fairly constant in the same patient over considerable periods of time In some cases it may be always within normal limits Merskey (1951a) has studied haemophilic families in which the coagulation times were relatively short and which he describes as cases of 'mild haemophilia' It should be emphasized that though there is a rough correlation between clotting time and severity a proportion of patients with relatively short clotting times may be severely affected A curious feature of the distribution of clotting times in 50 cases of haemophilia is the absence of values between 20 and 30 minutes (see Fig 41) The general conclusion to be drawn from these observations is that while a long clotting time

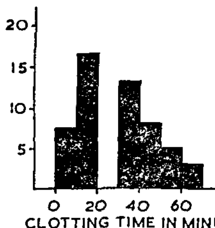


Fig 41 The clotting time by the Lee and White method carried out on 50 patients with haemophilia.

THROMBIN GENERATION

Measurement of the changing thrombin concentration of the blood (Macfarlane and Biggs 1953) or recalcified plasma (Pitney and Dacie 1953) during coagulation gives useful information in haemophilia and other conditions with defective thromboplastin formation The results obtained on normal whole blood are illustrated in Fig 42 and it will be seen that for the first three minutes after removal of the blood from the body there is no detectable thrombin Thrombin then begins to appear almost co-incidentally with the onset of fibrin formation and its concentration rapidly rises to a maximum and then as rapidly declines as the thrombin formed is destroyed by antithrombin or adsorbed on to fibrin It is probable that the initial delay phase is due to the time required for thromboplastin formation and that the rate and extent of thrombin formation is related to the amount of thromboplastin formed The addition of tissue extracts to normal blood almost abolishes the delay phase since the major time-consuming reactions in thromboplastin formation are by-passed but the rate and extent of thrombin formation is no greater than in the untreated blood

In haemophilic subjects marked changes in thrombin generation are usually observed In severe cases practically no thrombin can be detected at any time and the effect of adding varying proportions of A H C in the form of normal plasma is to produce corresponding changes in thrombin generation (Fig 43) From these results it can be seen that the effect of A H G deficiency is to greatly prolong the delay phase (thus lengthening the clotting time) and to reduce the rate and amount of thrombin formation In patients with short clotting times there is no reduction in the amount of thrombin formed and the delay is not sufficient to prolong the clotting time significantly (Figs 44 and 45) Deficiency of plasma factors concerned in thromboplastin generation other than A H G will give similar results but in platelet deficiency the pattern is different Thrombocytopenia produces a reduction of thrombin generated but unless the deficiency of platelets is almost complete it causes no delay in the onset of thrombin generation

PROTHROMBIN TIMES

Both the one-stage and the two-stage methods for estimating prothrombin give normal results in typical cases of haemophilia The reason for this is that coagulation with added tissue extracts

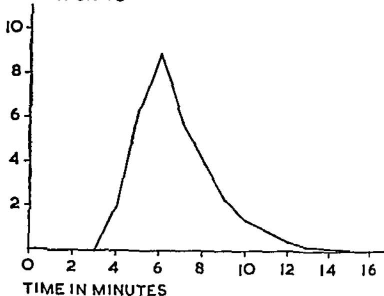


Fig 42 The thrombin generation test carri d out on normal whole blood The curve represents the average results of tests on 1_ normal subjects

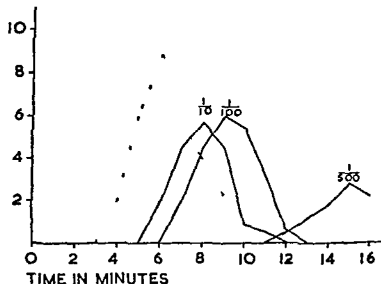


Fig 43 The thrombin generation test was carried out on the whole blood of a haemophilic patient whose Lee and White clotting tim was 42 mins No thrombin was generated unless some normal plasma was added The thrombin generated in the presence of 10 1 and 0 2 per cent of added normal plasma is shown

does not require the participation of the factors concerned with the early stages of coagulation except apparently Factors V and VII The one-stage prothrombin time test thus separates the deficiencies of Factors V VII and prothrombin in which the prothrombin times are abnormal from haemophilia Christmas disease Rosenthal s syndrome (P T A deficiency) and conditions due to inhibition of thromboplastin formation in which the prothrombin times are normal In these tests a considerable excess of tissue extracts is used Macfarlane and Biggs (1950) have described experiments suggesting that the reaction of haemophilic plasma to much smaller amounts of tissue extract may be less than that of the normal plasma but the interpretation of these observations is complicated by intrinsic thromboplastin formation in these slowly clotting systems by the possible occurrence of inhibitors in haemophilic blood and by the great sensitivity of haemophilic blood to calcium concentration

THE THROMBOPLASTIN GENERATION TEST

The observations of Biggs Douglas and Macfarlane (1953a b) indicated the existence of the complex system which forms thromboplastin in the blood after contact with a foreign surface and suggested that concerned in this process are at least two plasma factors (A H.G and Factor V) two factors found in serum (Christmas Factor and Factor VII) the platelets and ionized calcium A deficiency of these factors resulted in a delay or reduction in thromboplastin formation thus being more sensitively related to such deficiencies than were other tests of clotting function Not only was thromboplastin generation more sensitive it could be made a specific indicator of the type of deficiency

In the test devised by Biggs and Douglas (1953) prothrombin which would interfere with the measurement of thromboplastin, is removed from the plasma by adsorbtion on to $Al(OH)_3$ But this adsorbtion also removes Christmas factor and thromboplastin generation cannot proceed in its absence This factor but not prothrombin is present in serum so that a system composed of adsorbed plasma, serum platelet preparation, and calcium will generate a powerful thromboplastin after a few minutes of incubation thus thromboplastin being demonstrated by the addition of subsamples of the incubation mixture to normal recalcified citrated plasma By deriving the plasma or the serum or the platelets from

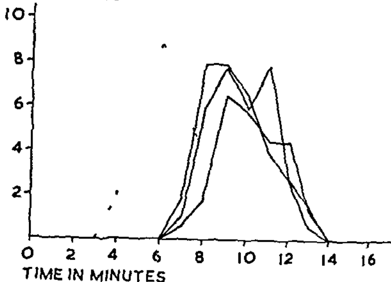


Fig 44 The thrombin generation test carried out on the whole blood of triplets who *suffered from haemophilia* *The Lee and White clotting time for these patients varied between* 10 and 14 minutes (normal 5 10 minutes) The discontinuous line represents the average results of tests on 12 normal subjects (Appendix IV 27)

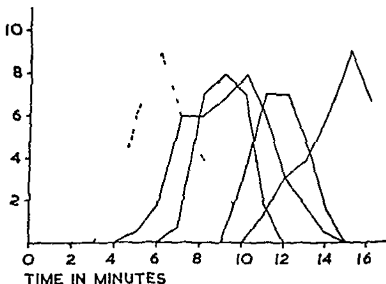


Fig 45 The thrombin generation test was carried out on four occasions on the whole blood of a haemophilic patient whose Lee and White clotting time varied from 14 18 minutes (normal 5 10 minutes) The *discontinuous line represents the average results of tests on 12* normal subjects.

THE COMPARATIVE VALUE OF LABORATORY TESTS

It is now possible to assess the relative value of the information supplied by the laboratory tests described in relation to clinical severity and the liability of any particular patient to bleed following trauma or surgery

The results of clotting time determinations show that in about 50 per cent of a series of 92 haemophilic patients a time of 30 minutes was exceeded Since haemophilia is the most common condition in which a greatly prolonged clotting time occurs such a finding is useful Of the patients with a short clotting time about 50 per cent were severely affected so that the occurrence of a short clotting time is no guarantee of a mild defect. On the other hand all the patients in this series who have survived surgery without A H G treatment had short clotting times

The results of the prothrombin consumption test show that in all cases with a long clotting time prothrombin consumption was abnormal of the cases with a short clotting time 12 patients had prothrombin consumption results within normal limits and of these 5 were moderately affected the remainder being mild cases Thus it is possible to say that if blood clotting times and prothrombin consumption tests are carried out all but a small proportion (5 out of 123 in this series) of the severely or moderately affected patients will show an abnormality by one or other of these tests In mild cases both tests may give normal results and the diagnosis of haemophilia cannot be established by their use alone

The thromboplastin generation test gave abnormal results in almost all the mild cases it being necessary to use the modified test previously mentioned to detect the abnormality in only two patients The results of the test are reasonably well correlated with clinical severity The A H G assay method gives a better index of clinical severity than the other tests

DIAGNOSIS

The diagnosis of haemophilia depends much on clinical as well as on laboratory findings It is not proposed to discuss these at length here since the differentiation of haemophilia from other coagulation defects is part of a systematic investigation which is described in Appendix II and the recognition of the existence of a haemorrhagic diathesis is a general clinical problem Both aspects have been dealt with elsewhere (Macfarlane and Biggs 1955)

the patient's blood and the remaining two components from normal blood, the location of any defect can be assigned to the plasma or serum or platelet components Thus a defect of A H G or Factor V is indicated by a defective plasma component and of Christmas factor or other serum factors by a defective serum component Further separation can then be made by means of the one-stage prothrombin time test which will be normal in Christmas disease and haemophilia and abnormal in Factor V and Factor VII deficiencies

Thus for the first time there is available a test for the haemophilic defect that does not rely on a supply of known haemophilic blood which in the past has sometimes proved to be very much of an 'unknown

In practice haemophilic patients with prolonged clotting times have always given grossly abnormal thromboplastin generation results and even in mildly affected cases some abnormality is found In general the amount of thromboplastin formed bears some relationship to clinical severity In exceptionally mild cases difficulty in diagnosis may arise and the test may be made more sensitive by comparing the thromboplastin generation of mixtures of known haemophilic plasma and normal plasma with that of mixtures of known haemophilic plasma and the patient s plasma (Biggs and Macfarlane 1956)

A H G ASSAY

The thromboplastin generation test can be modified to provide a quantitative estimate of A H G activity in a given sample The results can be expressed as a percentage of an average normal plasma activity or in terms of an arbitrary unit derived from the activity of a dried preparation of bovine A H G On this arbitrary basis 1 ml of normal plasma contains on the average 0 25 A H G units but there is a considerable variation from one normal individual to another ranging from about 50 per cent to 180 per cent of the average

The assay method has been used to study A H G levels in 39 cases of haemophilia In 25 cases no A H G was detectable and in the remainder the levels varied from 1 to 20 per cent In general it appeared that severely and moderately affected individuals have no measurable amounts of A H G and most mildly affected patients have between 5 per cent and 20 per cent though a small proportion of them had less than 5 per cent

While the majority of cases of haemophilia will present little diagnostic difficulty when carefully investigated there is a small proportion of cases in which atypical findings make classification difficult. Among these are included cases of otherwise typical haemophilia in which the bleeding time and tourniquet tests are abnormal cases in which a haemorrhagic state affects both males and females the males suffering apparently from typical haemophilia and the females from a variety of non-thrombocytopenic purpura and cases in which there seems to be a mild deficiency of both A G H and Christmas factor apparently inherited as a dominant character (Biggs and Macfarlane 1956) Finally a deficiency of A H G may occur very rarely as an acquired condition as in the case described by Joules and Macfarlane (1938) or due to the development of an inhibitor In von Willebrand s disease apparent A H G deficiency may also occur but otherwise the clinical and laboratory findings and the inheritance are distinct from haemophilia.

THE RECOGNITION OF FEMALE CARRIERS

Unfortunately there still does not exist any reliable method by which the female carrier of the haemophilic gene can be recognized other than her production of haemophilic children If it could be known with reasonable accuracy that any particular female in a haemophilic family was a carrier it is probable that a considerable reduction in the incidence of haemophilia could be achieved within two or three generations The uncertainty that exists at present encourages many affected women to gamble on the chance that they are free of the condition and to produce families with consequent propagation of haemophilia whereas if their condition was previously known they might have elected to remain childless Though Andraessen (1943) claimed to be able to recognize the carrier state by using the Bürker clotting time test, Merskey and Macfarlane (1951) were unable to confirm his results by this method or by using more sensitive tests for the haemophilic defect such as prothrombin consumption and ability to correct the clotting defect of haemophilic blood Though an occasional abnormal result was obtained in the group of known carrier females normal results were obtained so frequently that the tests were of no diagnostic value in any individual case It is possible that the improvements now being made in methods for the accurate assay of A H G may be able to reveal the heterozygous (carrier) state or that some other

Briefly the stages by which the diagnosis is established are as follows The history of abnormal bleeding from infancy particularly haemarthroses and deep tissue haemorrhages and the absence of purpura suggests a coagulation defect If other members of the family are affected, limitation to the male sex and evidence of a sex-linked inheritance are points strongly suggesting haemophilia or Christmas disease but it must be remembered that about one-third of all cases of these conditions will have no family history and that other coagulation defects may be inherited though usually as a dominant character

In severe cases of haemophilia the laboratory findings will be well defined and characteristic The normal platelet count tourniquet test and bleeding time usually will eliminate the vascular or platelet disorders as probable causes of the abnormal haemorrhage The lengthened clotting time may not always be found but is a finding of diagnostic value when present the prothrombin consumption test is abnormal in the great majority of cases These tests however are not specific being abnormal in a number of clotting defects The one-stage prothrombin time is a valuable test since it separates the clotting defects into two distinct groups The first group in which the prothrombin time is normal includes haemophilia Christmas disease and Rosenthal s syndrome (P T A deficiency) and thromboasthenia (qualitative platelet deficiency) The second group in which the prothrombin time is prolonged includes deficiencies of Factor VII Factor V prothrombin or of fibrinogen if this factor is much reduced Thus clotting defects with a normal prothrombin time can be narrowed down essentially to haemophilia and Christmas disease which will provide the great majority of cases and qualitative platelet deficiency and Rosenthal s syndrome which are extremely rare Conditions due to circulating anticoagulants which interfere with thromboplastin formation can usually be distinguished clinically since in most cases the condition is acquired, and can be recognized by laboratory tests described in Chapter XVII and in Appendix IV

The thromboplastin generation test will differentiate haemophilia from Christmas disease and if necessary qualitative platelet deficiency In mild cases of haemophilia the 'sensitized' thromboplastin generation test may be required to detect the A H G deficiency A H G assay is seldom required for diagnostic purposes but is useful in assessing the prognosis in a particular case

LOCAL TREATMENT

Increasing knowledge of the mechanism of coagulation and of the factors concerned in normal haemostasis has greatly improved local treatment of bleeding so that in many cases the haemorrhage from minor injuries or following dental extractions can be controlled without drastic measures or the need to give blood transfusions or A H G Briefly the principles of rational treatment are as follows coagulation at the bleeding point is promoted by the application of a suitable coagulant and at the same time temporary local pressure is applied which will arrest the blood flow and allow time for the formation of a firm clot re-inforcement of this clot is achieved by the use of an adsorbable dressing such as fibrin foam or alginate gauze and this is kept firmly but not tightly in place by appropriate mechanical means equally important any procedure that might devitalize or damage the tissues and thus delay healing is positively avoided

The use of physiological coagulants active in clotting haemophilic blood without damage to the tissues is essential to successful local treatment Some of the earliest were platelet extracts prepared by Fonio (1913) and lung extract used by Fischl (1916) Many commercial preparations based on these have been produced Unfortunately the coagulant activity of these substances was relatively low and their dilution with blood or saliva made them ineffective With the availability of coagulants of relatively enormous activity good results began to be obtained Russell s viper venom used by Macfarlane and Barnett (1934) has an intensely powerful thromboplastin-like activity and produces rapid coagulation of haemophilic blood even when diluted many million times Human or animal thrombin preparations of high activity have also been used (Lozner MacDonald Finland and Taylor 1941 Adams and Taylor 1943) Both Russell s viper venom and thrombin are now available commercially and either can be used in conjunction with an absorbable haemostatic dressing such as human fibrin fibrin foam gelatin sponge calcium alginate or oxidized cellulose gauze which can be left in situ until digested (see Chapter XIX) But the mere application of a coagulant dressing to a surface which is actually bleeding is usually ineffective since the coagulant is washed away from the bleeding points and merely produces useless clots elsewhere In order to produce effective haemostasis the clot must extend to the actual bleeding vessels and to achieve this the flow of blood must

method for detecting the abnormal gene may be devised Such an advance would be of the greatest importance in reducing the mental and physical distress now caused by haemophilia

PROGNOSIS

During the first year or so of life the severity of the haemophilic defect cannot be accurately assessed from clinical findings since the relatively protected life of a young child prevents the occurrence of many of the haemorrhagic episodes which may be suffered later As the child grows older and begins to sustain the knocks strains and injuries of everyday life the liability to external or tissue bleeding and to haemarthrosis will serve as a fairly accurate guide to the degree of disability If frequently repeated and caused by slight trauma the occurrence of joint or tissue haemorrhages indicate a severe defect and it is probable that much crippling and disability will finally result In such cases a normal life becomes hardly possible, and some special sheltered occupation may be only just within the patient's capacity All degrees of severity less than this are met with the mildest cases suffering abnormal bleeding only after severe trauma or surgical operations and being otherwise capable of leading a normal life Undoubtedly the best laboratory guide to prognosis is determination of blood level of A H G Cases with no detectable A H G are usually severely affected those with more than 5 per cent are almost invariably mild

TREATMENT

Before the recognition of the A H G deficiency in haemophilia treatment consisted of attempts to control excessive bleeding by local measures the administration of substances thought to assist the coagulation of the blood and a host of empirical and bizarre remedies Faced with persistent bleeding from some apparently trivial injury it is not surprising that drastic procedures were often undertaken which actually made matters much worse Wounds were cauterized by heat diathermy or chemicals tourniquets or tight dressings were left in position for long periods of time or tissues were tightly sutured over bleeding points in the hope of achieving haemostasis The result was almost always extensive tissue destruction and bleeding from a larger area than before or the formation of dangerous haematomas or actual gangrene following tight dressings or suturing and there is little doubt that many lives were lost as the result of these well-intentioned efforts

Surface Wounds The same general principles apply to the treatment of surface wounds Naturally any obvious arteries or veins which have been injured must be ligatured in the usual way but it is the persistent oozing from the minute vessels which cannot be controlled by the conventional means effective in normal subjects It is useless to sew up the wound in the hope that this will prevent haemorrhage It may prevent external bleeding but it almost always leads to haematoma formation which may reach enormous proportions and prove more dangerous than an external loss of blood It is better to leave adequate drainage of all wounds though gaping skin edges can be drawn together in order to avoid undue delay in healing Local haemostasis can often be achieved by packing with an absorbable dressing soaked with coagulant followed by the application of firm pressure over the wound for five minutes The dressing should then be kept firmly in place by bandages or adhesives we have found by experience that elastic one-way stretch bandages are most effective, and if necessary the pressure on the wound can be more evenly distributed by inserting a pad of sponge rubber between the turns

Internal Haemorrhage In most cases of internal bleeding only conservative treatment is practical Deep tissue haemorrhages may involve important structures and present a series of orthopaedic neurological and surgical problems It should be remembered that in haemophilia haemorrhage into the tissues is interstitial the blood infiltrating the tissue spaces widely and rapidly there are seldom localized collections of blood which can be drained and it is generally useless to incise or aspirate a haematoma in the hope of reducing its pressure on important structures Haemorrhage into the tissues of the tongue or throat often arising from local infection or injury can be rapidly fatal and prompt pharangeal or laryngeal intubation to preserve the airway may be required to save life (Macfarlane 1955)

Retroperitoneal haemorrhage can simulate an acute appendicitis or the perforation of an ulcer with confusing fidelity Haemarthroses are best treated by conventional orthopaedic means aspirations of the joint, and lavage with hyaluronidase or coagulants has been recommended but it is not clear that the results justify the risks of infection or provoking further bleeding Gastro-intestinal bleeding has been treated by giving coagulants by mouth but it is doubtful

be temporarily arrested so that a firm clot can form where it is needed This can usually be achieved by the application of local pressure exerted for at least five minutes and then cautiously relaxed The clot then formed will usually maintain haemostasis for several to many hours until destroyed by proteolysis or mechanically dislodged A repetition of the process may then be necessary

Dental Extraction One of the commonest causes of bleeding in haemophilics is tooth extraction Many patients have grossly decayed or infected teeth, the result of quite unjustified fear of even conservative dentistry on the part of the patient or dentist In consequence extractions which might have been avoided by prophylaxis become essential and without careful preparation and treatment may lead to prolonged and dangerous bleeding To avoid this a number of precautions must be taken The patient must be in *hospital with adequate facilities for local and* if necessary general treatment It is usually unwise to remove more than two teeth at a time unless general treatment is being applied and a dental plate should be carefully made which will fit accurately over the sockets of the teeth to be removed A general anaesthetic is preferable to local infiltration since injections devitalize the tissues and are also very apt to cause serious haematomata The greatest care should be taken to avoid unnecessary tissue damage during the extraction After the tooth is removed a small plug of fibrin foam or alginate gauze soaked in thrombin or Russell s viper venom solution is placed over the socket and held firmly in place by the operator s fingers for at least five minutes to promote the formation of a firm clot throughout the whole cavity The dental splint is then applied to keep this dressing in place with the minimum of movement and in most cases bleeding is thus controlled for 12 to 24 hours Reapplication of a fresh dressing may then be necessary due to lysis of the clot Tight plugging of the socket must be avoided as this delays healing everts the edges with enlargement of the wound and may cause a dangerous haematoma On no account must the gum margins be sutured together over the socket since this will lead to haematoma formation and has caused cases of fatal asphyxia from infiltration of the tissues of the throat with blood

If haemorrhage cannot be controlled by these simple local measures it is useless and harmful to apply any others that are more drastic and it is best to let the patient bleed while general treatment is being prepared

fraction by Patek and Taylor (1937) and concentration of this factor or antihaemophilic globulin (A H G) was achieved to the extent that the injection of 12 mgms of dried substance has been reported to produce a measurable effect on clotting time (Minot et al 1945) From that time most workers have recognized that the only effective method for improving haemostatic efficiency in haemophilia is the transfusion of fresh blood or the injection of concentrated A H G

The study of the relationship of blood A H G levels to the results of other laboratory tests and to actual haemostatic efficiency already described has clarified the problem of effective treatment From the observations of Brinkhous et al (1954) based on haemophilic dog blood and our own observations on a series of haemophilic patients investigated by the A H G assay method it can be concluded that a blood level of A H G at least 35 per cent of the normal is required to maintain haemostasis and to prevent bleeding from minor injuries and that 50 per cent is required to cover major injuries or operations Patients with less than 20 per cent of A H G have bled severely even though the clotting time and prothrombin consumption tests gave normal results below 10 per cent A H G the prothrombin consumption is usually abnormal but it is not until the level is below 2-5 per cent that the clotting time becomes prolonged (Biggs 1955 Biggs and Macfarlane 1956) Thus effective treatment must provide a blood level of A H G 10-25 times higher than that required to shorten the clotting time to normal It is likely that most of the many ineffective efforts to control bleeding reported in the literature were due to grossly inadequate dosage since shortening of the clotting time was then considered the criterion of effective treatment

Once the minimum haemostatic level of A H G is realized, the magnitude of the therapeutic problem becomes apparent It is actually very difficult to achieve this effective level by blood or plasma transfusion except in relatively mild cases of haemophilia Even if none of the A H G in normal blood is lost during collection or in the patient s circulation one would need to transfuse about 3 litres of blood or 1½ litres of plasma in an average adult in order to raise the blood level by 35 per cent In actuality considerable losses of A H G occur both during storage of the blood or plasma and in the patient s circulation It has been found by Langdale et al (1955) and Douglas (1955) that the blood level achieved by the

if any real benefit has been obtained Haematuria sometimes continues for long periods but usually the rate of blood loss, though apparently alarmingly high is actually low since as little as 1 per cent of blood in urine has by ordinary inspection the appearance of almost pure blood Many patients will continue to bleed for several weeks in this way despite repeated transfusion and stop bleeding as they began for no apparent reason

GENERAL TREATMENT

The general treatment of haemophilia has aimed at a correction of the clotting defect in vivo either as an emergency measure to control a current haemorrhagic episode or as a long term prophylactic therapy Many different lines of approach have been tried The most obvious has been the administration of coagulant substances of the thromboplastin type in general these have been ineffective since they have little coagulant activity in vivo a fact which fortunately prevented the intravascular coagulation or defibrination which might follow the injection of active thromboplastin Many empirical remedies have been tried often with temporary enthusiasm but not one has proved itself of definite value These include protein sensitization and anaphylactic shock (Vines 1920) egg white material (Timperley Naish and Clarke 1936) oxalate (Page Russell and Rosenthal 1940) venesection (Lawson Jackson and Gardner 1932) trypsin (Taynon 1944) histamine (Sanford Buttler and Kennedy 1951) ergot (Sturgeon and Friend 1951) and many others One of the most interesting attempts was the injection of female sex-hormones (Birch 1931) based on the observation that women do not suffer from haemophilia! Though some of the procedures mentioned produced a slight shortening of the clotting time they were clinically ineffective and it is now known that even the restoration of the clotting time to normal does not necessarily indicate a significant improvement in haemostatic efficiency

Transfusion of Blood and Plasma Although Lane (1840) seems to have been the first to demonstrate the beneficial result of blood transfusion in haemophilia general recognition of this effect was produced by the observations of Weil (1906) Transfusion of fresh plasma was found effective by Feissley (1924) and by Payne and Steen (1929) The active principle in normal plasma which in fact had been studied by Addis in 1911 was located in the globulin

human blood was hampered by lack of adequate methods of assay In consequence many preparations used therapeutically had little or no activity and even material produced by the carefully controlled Cohn process was unreliable (Alexander and Landwehr 1948a b Davidson Epstein Miller and Taylor 1949)

Recognition of the large amounts of blood which would be required to furnish enough A H G for even emergency treatment of haemophilia has encouraged investigations into the possible use of animal material Bovine blood has been shown to contain A H G activity and fractions of it were used as a local haemostatic by Pohle and Taylor (1938) Spaet and Kinsell (1953) and Lorand and Laki (1954) have described methods for preparing active fractions Recently animal A H G has been prepared and used in human cases in Oxford with success Bidwell (1955) has developed a method which produces bovine or pig globulin material with an A H G activity up to 2000 times as great as that contained in an equivalent weight of human plasma protein and more than 100 times as active as the best previous A H G preparations Injections of this animal A H G have been used to prevent bleeding after extensive dental extractions skin grafting operations major surgical operations and severe injuries and complete haemostasis was achieved (Macfarlane Biggs and Bidwell 1954 Fraenkel and Honey 1955) The dose given was controlled by blood A H G assays and daily injections were required if a safe level had to be maintained It was found necessary to raise the blood level of A H G to 30-50 per cent before any operation or traumatic procedure was carried out and if this was done haemostasis was maintained for some days even though the A H G level might decline below the safe level during this time At present there are practical difficulties involved in this treatment which render it unsuitable for general use The material is difficult to sterilize some samples will pass through a bacterial filter without much loss of activity others have to be sterilized just before use by ultra violet irradiation The dose has to be carefully controlled by assays of blood level, since the response to a given amount varies greatly from one patient to another and from day to day in the same patient Being a foreign protein animal A H G is antigenic and may cause anaphylactic reactions in some subjects who have been previously sensitized The duration of treatment is also limited by the antigenicity of the material because after about 10-14 daily injections of a particular preparation reactions begin

transfusion of a given amount of blood or plasma is 30-50 per cent of that which might be expected and even this is not maintained for more than a short time and declines by about 50 per cent in 4 to 8 hours A H G has only a short life in stored blood and the rate of its destruction is determined by factors at present unknown. In some samples of plasma stored at 4 C 50 per cent of the activity may be present after 10 days (Pitney and Dacie 1955) but in others most of the activity may disappear within twelve hours It has thus become the practise to transfuse blood within 2-3 hours of its collection and to prepare plasma by immediate centrifuging not by sedimentation Fresh plasma will preserve its A H G activity for days or weeks if kept frozen solid at −20° to −30 C fresh plasma dried lyophilically is sometimes active but on other occasions may lose much of its original activity Serum contains practically no A H G and the successes attributed to its use in the past are probably due to the fact that the patients so treated were cases of Christmas disease and not haemophilia

In cases of actual bleeding therefore blood transfusion can seldom provide enough A H G to promote efficient haemostasis not only because of the losses already mentioned but because blood (and A H G) is being lost in the form of haemorrhage Transfusion therefore has its main value in maintaining the blood volume the haemostatic effect being seldom more than an adjuvant to the patient s own resources The many reports in the literature of continual bleeding despite the transfusion of vast amounts of blood are thus not surprising

As a preparation for some very minor operation (such as tooth extraction) in a mild case of haemophilia the rapid transfusion of an amount of fresh plasma equal to about 1/5 of the blood volume may be quite effective if completed a few minutes before the actual extraction The temporary elevation of the blood A H G level at the time of trauma may be sufficient to cause firm natural clotting within a few minutes and this may provide effective haemostasis lasting for some days during which healing should be complete

Treatment with A H G In view of the almost impossibly large volumes of blood or plasma which are needed to restore and maintain haemostatic efficiency in a severely affected haemophilic concentrated preparations of A H G seem to provide the only practical form of therapy Unfortunately much of the earlier work on the separation and administration of A H G derived from

the genes in haemophilia and Christmas disease since mildly affected females not infrequently occur in the latter condition these having demonstrable clotting defects It is possible therefore that Christmas disease may be incompletely recessive possibly because a higher level of Christmas factor is required for efficient haemostasis than is the case of A H G so that the heterozygous female may be more liable to abnormal bleeding

LABORATORY FINDINGS AND DIAGNOSIS

The laboratory findings other than examination of thromboplastin generation are qualitatively similar in Christmas disease and haemophilia there is often a moderate prolongation of the clotting time abnormal prothrombin consumption but a normal 1-stage prothrombin time and normal platelet count bleeding time and tourniquet tests It is unusual for the clotting time to exceed 30 minutes in Christmas disease so that a grossly prolonged result suggests haemophilia The thromboplastin generation test locates the abnormality in the serum component in Christmas disease as against the plasma component in haemophilia In mildly affected cases the abnormality may be insufficient to give clear-cut results by the ordinary technique and a sensitized test as in the diagnosis of mildly affected haemophilic patients may be necessary It is obvious that accurate diagnosis is of extreme importance since Christmas disease will not respond to treatment with A H G

TREATMENT OF CHRISTMAS DISEASE

The principles for the local treatment of bleeding in Christmas disease are the same as those described in haemophilia Thrombin or Russell s viper venom are as effective coagulants of the blood in Christmas disease as in haemophilia

As regards general treatment there is little available information and we have had no experience with specific replacement therapy Theoretically such treatment should be easier than in haemophilia since Christmas factor is not consumed during coagulation and is relatively stable and its blood level should thus be more readily raised and maintained by transfusion The reports of Beaumont Caen and Bernard (1954) suggests that these theoretical considerations are borne out by fact. Haemorrhages can be controlled by fresh or stored plasma transfusions or by the transfusion of serum These authors claim that transfused Christmas factor remains in the

to develop which apart from their clinical unpleasantness reduce the therapeutic effect though a further therapeutic period can be instituted by changing to A H G derived from a different species of animal We have not been able to demonstrate the development of anticoagulants of the antibody type following animal A H G administration, though there is some evidence that repeated transfusions with human blood may induce the formation of such antibodies (Munro and Jones 1943, Munro and Munro 1946) and that the injection of Cohn s fraction I is particularly likely to cause it (Frommeyer Epstein and Taylor 1950) It is clear that the best hopes for the continuous treatment of haemophilia as in the case of treatment of diabetes with insulin and pernicious anaemia with Vitamin B12 rests on the possibility of producing an active but non-antigenic preparation which will correct the basic deficiency

CHRISTMAS DISEASE

The incidence of Christmas disease relative to haemophilia seems to vary a good deal from one country to another In the British Isles 21 families affected by Christmas disease have been separated out of a series of 174 families supposedly suffering from haemophilia giving a relative incidence of 11 per cent From information supplied to us by correspondents in Europe and the British Commonwealth it would seem that the incidence abroad may be higher the average being 32 per cent In certain areas the existence of very large families affected by Christmas disease as for instance the famous bleeders of Tenna' in Switzerland give an abnormally high incidence

CLINICAL FEATURES

The clinical features of Christmas disease are individually indistinguishable from those of haemophilia In our own experience there is a higher proportion of mildly affected patients as compared with haemophilia only 4 out of 11 cases being clinically severe similar observations have been made by Rosenthal (1954) As regards heredity the condition appears to be inherited like haemophilia as a sex-linked recessive character with a similar proportion (about 25-30 per cent) of cases without family history which probably represent mutations It is pointed out however by Pitney and Dacie (1955) that there may be a difference in the expression of

be determined if these cases in which the findings would seem to agree well with Rosenthal s conception of P T A deficiency are basically similar to the cases studied by him It is possible, however that in both groups of cases the explanation of the clinical and laboratory findings lies in a mild deficiency of both A H G and Christmas factor this combined effect being sufficient to cause abnormalities but apparent correction being obtained by the addition to the patient s blood of either factor Moreover the P T A of Rosenthal seems to have properties resembling both factors being sometimes associated with fibrinogen like A H G and sometimes with the β globulin like Christmas factor It might be expected from the very low incidence of these conditions that the chance of A H G and Christmas factor deficiency occurring in the same patient would be so small (1 in 10 million) that it might never occur In actuality well-defined cases have been described by Hill and Speer (1955) and Verstraete and Vandenbroucke (1956) and Koller (1954) reports a single family in which haemophilia has affected one member and Christmas disease another There are possible explanations for these associations It may be that Christmas factor and A H G are physiologically linked in some way which is not yet apparent and that haemophilia and Christmas disease are variants of the same basic disorder or it may be that there is some even more remote genetic linkage or it may be that mild deficiencies of either factor are much more common than we suppose but are not sufficient to cause any clinical abnormality unless by chance they occur in combination

VON WILLEBRAND S DISEASE

Though it has been called pseudo haemophilia by some authorities von Willebrand s disease has no clinical or genetic resemblance to haemophilia, and since it more closely resembles the purpuric conditions it is described in Chapter XVI Until recently, the evidence suggested that the haemorrhagic tendency was due solely to some abnormality of the superficial capillaries There were a number of reports of defective coagulation deficient clot retraction and deficient prothrombin consumption in cases clinically resembling von Willebrand s disease and these defects could be traced to a qualitative deficiency of the platelets which though present in normal numbers did not react normally in thromboplastin genera-

recipient s serum for 2-3 weeks White Aggeler and Emery (1953) have attempted to purify the factor and it is probable that concentrated material with Christmas factor activity will soon be available for clinical use

P T A DEFICIENCY (Rosenthal s Syndrome)

Rosenthal (1954) has reviewed the evidence which leads him to conclude that in a certain number of patients with a haemophilia-like condition there is a deficiency of a factor distinct from A H G and Christmas factor and which is concerned with plasma thromboplastin He has called this factor P T A ' (plasma thromboplastin antecedent) the properties attributed to this and the evidence on which its existence is postulated have been discussed in Chapter VIII where P T A *deficiency is referred to as Rosenthal s syndrome*

The clinical findings in Rosenthal s syndrome are similar to but usually milder than those of haemophilia or Christmas disease In only 1 of Rosenthal s 6 cases was there any bleeding into joints and the main abnormality was prolonged haemorrhage after tooth extraction or tonsilectomy The condition affected both males and females and there was evidence of a non-sex linked possibly dominant inheritance The laboratory findings included moderate prolongations of the coagulation time deficient prothrombin consumption with normal platelet counts bleeding time and tourniquet tests and a normal prothrombin time The finding which distinguishes these patients from cases of haemophilia and Christmas disease is the corrective effect on their blood of either serum or $BaSO_4$-treated plasma Haemophilic blood is corrected by the plasma and not by the serum Christmas disease blood is corrected by the serum not by the plasma P T A -deficient blood is corrected by both the plasma and the serum

We have studied three patients in whom the dominant inheritance symptoms and laboratory findings are very similar to those recorded by Rosenthal Dreskin and Rosenthal (1953) and Rosenthal (1954) When examined by the thromboplastin generation test one of these patients appeared to have a mild deficiency of A H G and Christmas factor, a second case seemed to fluctuate from time to time between the two conditions and the third appeared to be a very mild example of Christmas disease It has already been pointed out that it cannot

PLATELET DEFICIENCY

Soon after the recognition of the platelets as one of the formed elements of the blood it was realized that they were concerned in some way with blood coagulation Hayem (1878) and Bizzozero (188-) both stated that they probably released some factor which was necessary for fibrin formation Delezenne (1897) LeSourd and Pagniez (1909) Gratia (1914) and Hess (1917) were all able to show that partial or complete removal of the platelets by centrifuging caused a marked lengthening of the clotting time of the supernatant plasma even when in contact with glass and in the presence of ionized calcium It is true that Delezenne and some later workers were dealing with the blood of birds and amphibia in which the nucleated platelets (thrombocytes) being less fragile than mammalian platelets can be more easily removed intact by centrifuging The almost complete inhibition of coagulation produced by centrifuging this type of blood can be reproduced with mammalian blood only under exceptional circumstances

Cramer and Pringle (1913) and Eagle (1935a) considered that the incoagulability of mammalian plasma after filtration through Berkefeld filters was due to removal of the platelets Such experiments are not easy to interpret since it is now known that such filtration causes a complex and variable adsorption of different clotting factors depending on the rate of filtration the amount of plasma passing through a given area of filter pH and the ion concentration of the filtrate and the presence of capillary active substances which greatly affect adsorbtion Though Eagle (1935a) was able to show that the clotting time of recalcified filtered plasma was dependent upon the number of platelets subsequently added it is possible that this coagulant effect was due not to the added platelets only but to plasma factors contaminating them In the case of haemophilic blood Quick (1942) found that partial removal of platelets by centrifuging had a considerable lengthening effect on the clotting time of the recalcified plasma and proposed that this effect might be used as a test for haemophilia In practice it has been found that the effect is not specific and is not marked in those cases of

tion There still remained the majority of cases clinically identified as examples of von Willebrand's disease in which both the platelets and the clotting system appeared to be normal It has recently been observed however that there is an apparent deficiency of A H G in such cases as judged by the inability of their blood to correct the haemophilic defect (Larrieu and Soulier 1953 Alexander and Goldstein 1953) We ourselves have found by the assay method considerable deficiency of A H G in 7 cases otherwise typical of von Willebrand s disease The surprising fact is that in these cases all other tests of clotting function were normal including prothrombin consumption and clot retraction tests and that the patients showed none of the typical manifestations of haemophilia These apparent anomalies must be resolved before the physiological importance of A H G in haemostasis can be understood

SUMMARY TO CHAPTER XV

Historically haemophilia was recognized as a bleeding disease of males inherited as a sex-linked recessive characteristic Strict adherence to the classical theory of blood coagulation and lack of suitable techniques delayed the understanding of the clotting defect in haemophilia Following the introduction of silicone surfaces and of the thromboplastin generation test it was shown that the defect was a failure of normal blood thromboplastin formation for which A H G (the factor deficient in haemophilia) is an essential component Correction of the haemostatic defect in haemophilia involves raising the blood A H G level to more than 30 per cent which is in practice very difficult unless concentrated A H G preparations are available Local measures for the treatment of bleeding must always take account of the fact that haemophilic bleeding is controlled by healing and measures which delay healing will prolong bleeding

The use of the thromboplastin generation test has also made it possible to study other causes of deficient thromboplastin formation of which Christmas disease (P T C deficiency haemophilia B) is the most important Failure to distinguish between haemophilia and the clinically identical Christmas disease has in the past given rise *to much confusion*

mm and below these figures there was a considerable falling off in prothrombin consumption They also observed that the plasma could be made incoagulable by a partial reduction of the platelets in cases of thrombocytopenia and in patients receiving heparin or dicoumarol

Merskey (1950a) has confirmed the effect of the platelets on the prothrombin consumption of recalcified citrated plasma He observed that prothrombin consumption was almost absent in eight cases of naturally occurring thrombocytopenia and in the plasma of normal subjects it was reduced to zero by centrifuging We have also observed that the thrombin generation test described in Appendix IV 27 is greatly affected by the number of platelets present in the blood or plasma Figure 46 shows a series of thrombin generation curves obtained with different concentrations of platelets and it will be seen that the amount of thrombin produced is dependent upon platelet concentration though the time at which thrombin generation begins is not greatly affected

THE MECHANISM OF THE COAGULANT ACTION OF THE PLATELETS

It was at first believed that the platelets released into the plasma some soluble factor capable of initiating thrombin generation The nature of this factor was not determined but was assumed by some workers to be thromboplastin and by others to be prothrombin The latter hypothesis was disproved by Bordet and Delange (1912) and it is now generally agreed that the platelets have some thromboplastic activity though most workers have considered that it is relatively feeble (see however Chapter VI and below) It is certainly qualitatively different from the thromboplastic activity of tissue extracts since these are capable of clotting haemophilic blood whereas the addition of plasma-free normal platelets has no such effect This activity seems to be associated with the platelets themselves or with demonstrable platelet fragments and the existence of a soluble platelet factor has not been shown Platelet activity however does seem to depend on the physical changes which they undergo on contact with a foreign surface and in the presence of calcium Wright and Minot (1917) described the viscous metamorphosis of the platelets during coagulation and Tait and Burke

haemophilia in which the clotting time is near normal these being the cases in which diagnostic help is most required

The use of silicone-coated syringes needles and glassware has greatly facilitated the removal of platelets undamaged by centrifug ing Patton, Ware and Seegers (1948) using this technique were able to produce samples of dog plasma which collected without anticoagulant and centrifuged at high speed remained fluid for 72 hours in glass at room temperature Brinkhous (1947) demonstrated that the coagulation time of human plasma in glass can be prolonged to many hours by removal of the platelets by centrifuging in silicone-coated apparatus Quick Shanberg and Stefanini (1949a b) showed that while the number of platelets within a wide range does not greatly affect the ordinary coagulation time it closely con-

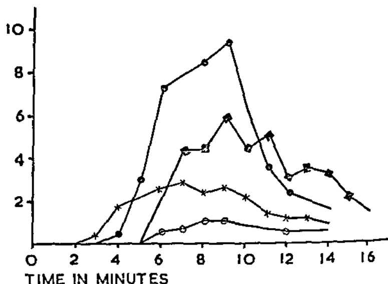


Fig 46 *The thrombin generation test was carried out on normal plasma prepared by centri fuging* normal blood collected into a silicone coated container The number of platelets in the plasma was varied by mixing plasma samples which had been centrifuged at different speeds The proportion of platelets present directly affects the amount of thrombin formed O—O platelet poor plasma ●—● platelet rich plasma ×—× 10 per cent of platelet rich plasma. ■—■ 20 per cent of platelet rich plasma

trols the amount of thrombin generated This observation was confirmed by Conley Hartmann and Morse (1949a) who found that prothrombin consumption showed a significant decrease even when the platelets were reduced from 350 000 to 260 000 per cu.

contaminated with other clotting factors or of thrombin derived from one animal species and containing a natural agglutinin for platelets derived from another

Whatever the effect of thrombin on platelets it is now clear that other plasma factors are intimately concerned with the platelets in the production of blood thromboplastic activity From the work of Brinkhous (1947) and Quick (1947) it can be inferred that one of these factors is A H G They showed that both platelets and A H G were required for prothrombin conversion though the nature of the reaction between A H G and the platelets was not established Quick believed that A H G was *the precursor of plasma thromboplastin* (thromboplastinogen) which was activated in some way by the platelets Brinkhous suggested that A H G was a 'thrombocytolysin which lysed the platelets, and allowed the escape of a thromboplastic factor from them

More recent work on thromboplastin generation has established the importance of the platelet-plasma reactions if not their nature It seems probable that not only A H G but the Christmas factor and calcium are required together with normal platelets during the initial stages of thromboplastin formation (see Chapter VI) A reduction in the number of platelets causes a corresponding reduction in the amount of thromboplastin formed but, unlike a reduction in A H G or Christmas factor does not cause much delay in the time required for its first appearance This supports the view that the platelets contain a thromboplastic factor or at least its precursor that the plasma factors undergo some time-consuming reaction which leads to its release It also explains the normal coagulation time usually observed in thrombocytopenic blood Unless there is a complete absence of platelets and platelet fragments which seldom occurs in practice thrombin generation begins at approximately the normal time but the amount of thrombin generated and the amount of prothrombin consumed is reduced in proportion to the reduction in platelets

Recent work by Bergsagel (1956) seems to have confirmed these views He has observed both the occurrence of viscous metamorphosis and the development of thromboplastic activity in preparation of platelets and various combinations of clotting factors From this it appears that the sequence of events is as follows After contact with a foreign surface a reaction takes place between A H G Christmas factor and calcium, with the formation of an intermediate

(1926) gave a detailed account of the changes in their appearance during this process, consisting of the extrusion of clear blebs or spherules followed by disintegration These spherules were described as travelling through the plasma and the first filaments of fibrin seemed to appear behind them as they moved In blood which is rendered incoagulable by oxalate citrate heparin or absence of contact with a foreign surface these changes are not observed

The nature of the metamorphosis and disintegration of the platelets is quite obscure Apitz (1939) believed that the initial agglutination was brought about by a deposit of fibrin on the platelet surface but Pinniger and Prunty (1946) have been able to show that active platelet agglutination occurs in the blood of a patient with complete absence of fibrinogen in which no fibrin layer could have been formed Wright and Minot (1917) believed that the platelet changes called by them 'viscous metamorphosis were brought about by some agent in the plasma associated with the globulin fraction and resembling prothrombin They considered that this activating agent was not thrombin itself but Quick (1951a) believes that thrombin is a most important factor which produces these platelet changes and which potentiates their thromboplastic activity Fonio (1923) and Zatti (1948) have observed that thrombin is capable of causing platelet agglutination and lysis On the other hand Budtz-Olsen (1951) has found that a mixture of platelets thrombin and fibrinogen does not produce retractile blood clots Clot retraction is intimately concerned with platelet activity and such activity was not induced by thrombin alone but was induced if prothrombin was present suggesting that something is formed during prothrombin conversion which has an activating effect on platelet metamorphosis These observations were borne out by the work of Ellicot and Conley (1951) though these authors in addition found that clot retraction could be induced in a platelet-thrombin-fibrinogen mixture not only by the addition of plasma or serum but by albumin of human or bovine origin or even gum acacia More direct evidence has been provided by Bergsagel (1956) who during his study of the effect of plasma factors on the viscous metamorphosis and thromboplastic action of the platelets found that homologous thrombin had no effect in potentiating these changes It is possible that some of the previous reports of the activity of thrombin in producing platelet agglutination or lysis may be due to the use of thrombin

that the opalescent plasma obtained from animals during the digestion of a heavy meal clotted normally despite the removal of platelets Poole and Robinson (1956) and Robinson and Poole (1956) have shown that the addition of chylomicra to platelet-poor plasma shortens the calcium time and increases the yield of thrombin Russell s viper venom which may be analogous to one of the thromboplastin-forming factors will not clot plasma from which both platelets and chylomicra have been removed but will do so if either are restored or if lecithin preparations are added They have also shown that ethanolamine phosphatide which is a constituent of platelets chylomicra and most laboratory preparations of lecithin will shorten the clotting time of platelet-poor plasma and potentiate the venom and may thus be at least a constituent of the platelet factor

By various methods of fractionation Van Creveld and Paulssen (1951b 1952) and Stefanini and Campbell (1954) have identified other platelet factors influencing coagulation Platelet factor 1 accelerates the conversion of prothrombin by thromboplastin and is probably identical with Factor V which may have been adsorbed from the plasma Platelet factor 2 accelerates the thrombin fibrinogen reaction Platelet factor 3 was considered by Van Creveld and Paulssen (195-) to have two actions one as an active thromboplastin the other as an antiheparin From the experimental data provided it seems very likely that factor 3 consists of the granules studied by Bergsagel (1956) and Deutsch (1954) claims to have separated the platelet thromboplastic factor from the antiheparin which he calls factor 4 Seegers (1954) has also studied the reaction of platelet factor 3 with plasma constituents including A H G and has applied the name threone to the thromboplastic activity which as observed by many other workers develops in such systems

In addition to the increasing knowledge of the importance of the platelets in coagulation there is now a growing recognition of their function as transporters of biochemically active substances such as 5-hydroxy tryptamine histamine adrenalin and adenosine triphosphate (Weil-Malherbe and Bone 1954 Zucker Friedman and Rapport 1954 Born 1956) The significance of the presence of these substances in the platelets in some cases in remarkably high concentration cannot yet be assessed but it is obvious that such factors may have an important bearing on the at-present mysterious relationship between the platelets and the vascular factors concerned

product This intermediate product then causes morphological changes in the platelets consisting of swelling active pseudopodia formation agglutination and finally fusion these changes being characteristic of those described as 'viscous metamorphosis During these changes granules which were originally visible at the centre of the platelet body move towards the periphery and are discharged into the surrounding plasma These granules can be collected by high speed centrifugation, and are an extremely active thromboplastic material since in the presence of Factor V they will cause the clotting of recalcified plasma in 4-8 seconds In the absence of A H G or Christmas factor or calcium neither viscous metamorphosis of the platelets nor the release of the active granules takes place It is not clear if these reactions lead to some qualitative change in the platelet granules but once discharged in this way they have a thromboplastic activity not possessed by extracts of platelets mechanically or chemically obtained and are capable of activating prothrombin in the absence of A H G or Christmas factor but require apparently the presence of Factor V

Though the participation of the platelets in thromboplastin formation and thus in normal coagulation is of great physiological importance there is evidence that in certain circumstances they may not be essential In a few experiments already quoted non-clotting plasma has been obtained by simple removal of the platelets but it is more often found that even prolonged high-speed centrifuging sufficient to separate all platelets and platelet fragments does not do more than slow the coagulation of the plasma so treated and reduce prothrombin consumption Some authors such as Conley Hartmann and Morse (1949a) interpret their failure to produce incoagulable plasma by centrifuging as evidence for the existence of a plasma thromboplastin presumably in solution It is possible that the platelet granules may not always be thrown down by the centrifuges used but it is also possible that there are other sources in the plasma of a thromboplastic factor similar to that of the platelets Nolf (1938) points out that lymph which contains no platelets will clot and in this case a thromboplastic factor might be derived from the tissues Shinowara (1951) obtained blood thromboplastin from blood cells in general and Quick et al (1954) have shown that red cells possess a thromboplastic activity There is increasing evidence that plasma lipoid and chylomicra may also act in thromboplastin generation Cramer and Pringle (1913) observed

troubles though there are repeated exacerbations and partial remissions lasting for variable periods of time Spontaneous recovery is rare and in our experience is more likely to occur in children than in adults

In the acute form described by Hirsch and Dameshek the disease is limited to one or two episodes of haemorrhage lasting only a few weeks or months and usually followed by spontaneous recovery Some of the acute cases described by these authors clearly belong to the secondary thrombocytopenic purpura group as definite aetiological factors were present

The blood findings in this condition show a variable degree of anaemia and possibly iron deficiency as a result of haemorrhage The platelets are reduced being usually below 100 000 and sometimes too few to count Clinically there may not be a very close correlation between the number of platelets and the severity of the bleeding condition and the many attempts which have been made to define some threshold level below which haemorrhage is likely to occur have not in practice been found to succeed

Clot retraction is usually diminished or absent It has been maintained by some workers that the clots formed in thrombocytopenic blood are less robust than normal but Budtz-Olsen (1951) has measured the mechanical strength of clots produced in platelet containing and platelet free plasma and has found no significant difference The coagulation time as ordinarily measured is usually normal but some observers have reported a significant lengthening of the coagulation time of recalcified plasma as measured by electrophotometric methods (Nygaard et al 1940 Croizat Favre-Gilly Perrin and Durant 1949) All observers are agreed that there is a considerable decrease in prothrombin consumption which is closely related to the number of platelets available (Merskey 1950a) No other significant abnormalities of the peripheral blood have been described The bone marrow in typical cases shows the presence of a normal (Diggs and Hewlett 1948) or increased (Dameshek and Miller 1946) proportion of megakaryocytes These observers agreed that there is a significant arrest in the maturation of these cells there being a greater proportion of the more immature forms with a deficiency of the mature platelet producing cells Robson (1949) while agreeing that platelet production appears to be deficient in the marrow of cases of thrombocytopenic purpura has not confirmed the preponderance of immature cells described by the previous

in haemostasis and in the development of spontaneous capillary haemorrhages

NATURALLY OCCURRING THROMBOCYTOPENIA

A reduction in the number of circulating platelets may occur as a result of many different conditions and diseases and also as an idiopathic or essential thrombocytopenia Thrombocytopenia is usually but not always associated with the clinical condition of purpura haemorrhagica in which the patient suffers from prolonged oozing from superficial injuries or from apparently intact mucous membranes and develops petechial or ecchymotic haemorrhages in the skin either spontaneously or as a result of pressure or minor trauma In these patients the bleeding time test that is the time required for cessation of haemorrhage from a small puncture wound is greatly prolonged, and the tourniquet test is positive indicated by the appearance in the skin of the forearm of petechial haemorrhages below a constricting pneumatic cuff applied to the upper arm

Descriptions of such cases as these go back to very early times Lusitanus (1556) describes a flea-like disease without fever and Rivierous (1658) a case in which there were 'purple spots like flea bitings due as he very rightly supposed to blood 'coming out of the capillary veins under the skin The prolonged bleeding time was observed by Stoker (1823) and the failure of clot retraction which is characteristic of thrombocytopenia was observed by Duncan (1882) The reduction of the platelets in such cases was described by Krauss (1883) and by Denys (1887) Werlhof (1775) had already described what is almost certainly the essential form of thrombocytopenia and his name is by custom now associated with this particular condition

ESSENTIAL THROMBOCYTOPENIA (WERLHOF'S DISEASE)

Essential thrombocytopenic purpura is a disease of at present unknown aetiology characterized by the manifestations already described and by a long continued reduction in the number of circulating platelets It may appear at any age but is commonest in young adults particularly women Hirsch and Dameshek (1951) recognize a chronic and an acute form of the condition In the former variety which is the commoner (55 out of 81 cases belonged to this group) the patient is seldom completely free of haemorrhagic

episode but it can also be employed for longer periods of time in cases in which splenectomy is contra-indicated or has failed to produce a cure Cortisone has been found more effective than A C T H by Adamson et al (1953)

There have been reports that the administration of 5-hydroxytryptamine has produced clinical improvement in cases of thrombocytopenic purpura (Ravetta 1955) It is too soon however to assess the extent or duration of the improvement claimed

SECONDARY THROMBOCYTOPENIC PURPURA

Thrombocytopenic purpura may occur as a secondary phenomenon in a number of acute infections as a result of poisoning by chemicals and drugs in sensitive individuals after exposure to certain substances to which they have developed antibodies and in any condition in which the bone marrow is destroyed or replaced by some other tissue

A complete list of these conditions and the circumstances in which thrombocytopenia may arise is given with references by Wintrobe (1951) It need only be said here that many acute bacterial or virus infections may cause isolated cases of thrombocytopenic purpura The haemorrhagic forms of small-pox measles typhus and meningococcal septicaemia are familiar clinical entities which may be due to the development of this type of purpura Various chemical preparations used in industry or therapeutically may produce purpura though the susceptibility of different individuals to these agents seems to vary very widely Among those listed by Wintrobe are benzol dinitrophenol iodine bismuth gold salts quinine quinidine tridione phenobarbitone sedormid D D T sulphonamides sodium salicylate thiourea organic arsenicals streptomycin and nitrogen mustards The condition may also occur as a result of sensitization to a number of different allergens among foodstuffs and following severe burns Destruction of the bone marrow by X-rays or radium irradiation and as a result of acute leukaemia aplastic anaemia and replacement by new growths will cause thrombocytopenic purpura

Thrombotic Thrombocytopenic Purpura

About 50 cases of a fatal condition have been described in which the patient usually a female develops an acute thrombocytopenic purpura abnormal bleeding haemolytic anaemia and transitory

workers In Chapter XII it was emphasized that the platelet deficiencies would be described only in their relation to coagulation The most important feature of Werlhof s disease the capillary defect indicated by the long bleeding time and positive tourniquet test cannot therefore be discussed

Effects of Treatment

As might be expected many forms of treatment have been applied, including stimulants of marrow function transfusion of whole blood and platelet suspensions cortisone and A C T H therapy and splenectomy Of these splenectomy still seems to be the only procedure which gives permanently good results Hirsch and Dameshek (1951) report success in 63 per cent of their cases and Giannini (1952) gives an estimate of 80 per cent from a survey of the literature The beneficial effects of splenectomy in most cases appear before there is any significant rise in the platelet count since the haemorrhagic manifestations may cease the bleeding time and tourniquet test may become normal within a few hours of the operation whereas platelets may not rise significantly for several days (Macfarlane 1941 Robson 1949 Giannini 1952) In most cases a rise in the platelet count to normal occurs and it may be maintained so that there is a complete clinical and haematological cure In a few cases the clinical cure may persist though the platelet count after a transitory rise may relapse to its original low level In the remainder either no beneficial effects are observed or after a temporary remission the condition returns with its original severity

Stefanini et al (1952) and other workers including Minor and Burnett (1953) have obtained temporary clinical improvement by transfusing platelet-rich blood or concentrated preparations of platelets and their findings suggest that the method might be used to improve haemostasis temporarily during a surgical operation such as splenectomy or during an acute haemorrhagic episode

More prolonged benefit may be obtained from the use of A C T H or cortisone The administration of these substances may produce a marked clinical improvement with a reduction of spontaneous bleeding and the restoration to normal of capillary fragility and bleeding time tests but not necessarily any increase in the platelet count (Pariser and Wassermann 1954 Nordenson and Havermark 1952) Again this form of treatment may be used in an emergency to cover a surgical operation or to control an acute

who have recovered from this particular type of purpura results in the appearance of a local patch of haemorrhage and of decreased capillary resistance without a general reduction in the number of circulating platelets He therefore considers that sedormid acts directly upon the capillary endothelium to form a complex susceptible to the damaging effects of the circulating antibody This assumes that the capillary endothelium is antigenically similar to the platelets an assumption supported by other observations such as the fact that specific antiplatelet serum injected locally into the skin produces a spreading area of haemorrhage which can only be due to a direct action upon the vessels (Tocantins 1936b) It is likely that other drug and chemical sensitivities behave in a similar manner to that of sedormid Bolton and Young (1952) have been able to demonstrate the lysis and agglutination of platelets by quinine quinidine and sulphonamide in patients recovering from purpura due to these respective drugs

In animal experiments results have been obtained which suggest that the haemorrhagic manifestations of purpura and the thrombocytopenia which usually accompanies it are not directly related The injection of relatively non-toxic substances such as gelatin (Roskam 1922) or agar-serum (Bedson 1922) can produce severe thrombocytopenia without producing purpura On the other hand a relatively specific anti-endothelium' serum will produce haemorrhagic purpura without reducing the platelets (Clarke and Jacobs 1950) Such observations taken in conjunction with those of Ackroyd may clarify many of the perplexities of thrombocytopenic purpura

In a proportion of the cases of secondary thrombocytopenia it is reasonable to suppose that an abnormal platelet antigen is produced by the action of the drug or chemical and that the patient produces a specific antibody to this complex which results both in thrombocytopenia and in capillary damage In these cases and others in which the toxic factor has not yet been demonstrated either the platelet or the capillary damage may predominate so that thrombocytopenia without bleeding or bleeding without thrombocytopenia may occur In cases of aplastic anaemia in which thrombocytopenia is the result of a more or less complete cessation of marrow activity it is possible that an agent which has destroyed the blood forming elements in the bone marrow has also damaged the capillaries In cases of leukaemia and metastatic involvement of the bone marrow

focal neurological signs At post mortem examination widespread capillary thrombi composed of platelets are found these being probably responsible for the neurological changes There is also evidence that endothelial damage precedes and probably causes, the accumulation of platelets in the small vessels and may deprive the blood stream of platelets and produce the thrombocytopenia No cause for the endothelial damage and haemolysis has been discovered but there are resemblances to the changes observed in the Schwartzmann reaction a state produced in sensitized animals by the injection of an allergen The condition is described and the literature reviewed by Singer Motulsky and Shanberge (1950) Symmers and Barrowcliff (1951) and Adelson et al (1954)

THE MECHANISM OF THROMBOCYTOPENIA

SECONDARY THROMBOCYTOPENIA

The causes of thrombocytopenia are naturally easier to investigate in the secondary variety of the condition than is the case in essential thrombocytopenia The most illuminating work in the whole subject has been that of Ackroyd (1949a b 1951 1955) who has studied the thrombocytopenia occurring in patients taking sedormid This substance has long been known to produce purpura in certain people and its fairly wide use as a sedative has provided a number of cases Ackroyd has been able to show that if sedormid is added in vitro to the blood of patients who have recovered from sedormid purpura the platelets are agglutinated and lysed This platelet lysis required complement and complement is fixed during the process Alone the platelet free plasma of the patients has no effect on either their own or on normal platelets but in the presence of complement and sedormid is capable of lysing both normal platelets and the patient's platelets In the absence of complement only platelet agglutination takes place The analogy to haemolysis and haemagglutination by specific antibodies is at once apparent Ackroyd suggests that a sedormid-platelet complex is formed which is antigenic and to which certain people produce antibodies These antibodies are incapable of lysing or agglutinating normal platelets but will react with platelets in the presence of sedormid He has also been able to show that the application of sedormid to the skin of patients

thrombocytopenia caused a fall in the platelet count and development of purpura in normal recipients

Following these observations there have been many reports of the demonstration of platelet agglutinins and in some cases of platelet lysins in the sera of patients with idiopathic thrombocytopenic purpura Harrington et al (1953) Stefanini et al (1953) Tullis (1953) and Stefanini (1955) report investigations on a total of over 200 patients in which platelet antibodies were demonstrated in from 12 to 50 per cent of the cases in the various series examined These antibodies varied considerably in their activity and specificity In most cases they seem to have been autoagglutinins active against the patient s own platelets in some instances normal platelets from certain donors but not from others were agglutinated in a few instances (Tullis 1953 Dausset Delafontaine and Fleurio 1952) the sera were shown to cause lysis of normal platelets if complement was present in other cases though no agglutination was produced by the sera it was shown that coating of the platelets had taken place by means of various modifications of the Coombs test (Flückiger Hüssig and Koller 1954)

The different reactions of different platelet samples to the same agglutinin naturally suggests antigenic differences in the platelets themselves There have thus been many attempts to group platelets antigenically Harrington et al (1953) record results that suggest the existence of at least twelve platelet groups and Stefanini et al (1953) considered that they could distinguish six The latter authors also provided evidence that two iso-agglutinins exist in normal plasma indicating four main platelet groups Though they could not relate these to the ABO red cell groups other authors (Gurevitch and Nelken 1954 and Morreau and André 1954) considered that platelet groups corresponding to the ABO system could be demonstrated

Against this growing mass of evidence for the existence of specific platelet antibodies must be set the difficulties and fallacies of the techniques used to study them Platelets are much more difficult to handle than red cells as regards the observation of specific agglutination Platelets tend to agglutinate spontaneously since this is their normal behaviour during the early stages of coagulation This agglutination is not easy to prevent by even careful collection technique and the use of special anticoagulants Even if control systems show no agglutination, positive results may not necessarily

this hypothesis is less convincing It is known however that haemolysis is a feature of many such cases and if the rapidly growing malignant tissue secretes lysins responsible for the destruction of normal tissue and red cells these may also damage the capillaries in general

IDIOPATHIC THROMBOCYTOPENIC PURPURA

During the past three years there has been a rapid development of the idea that a proportion at least of the cases previously classified as idiopathic' thrombocytopenia may be due to the development of agglutinins or lysins active against the patient's own platelets and capable of producing thrombocytopenia by a process analogous to the action of red cell antibodies in haemolytic anaemia The view that platelet-damaging substances might be concerned in idiopathic thrombocytopenia goes back for many years Frank (1925) suggested such a mechanism and Troland and Lee (1938) claimed to have shown that extracts of spleen tissue from cases of thrombocytopenia caused a fall in the platelet count when injected into rabbits an observation which was carried further by Torrioli and Puddu (1938) who found that these extracts also damaged megakaryocytes These observations however did not suggest an immunological basis for the anti-platelet activity though they supported the concept of hypersplenism which postulates a rather mysterious over-function of the spleen in destroying platelets and inhibiting their production which has been current in recent years Bedson (1922) was probably the first to introduce by his experiments with antiplatelet serum the idea that thrombocytopenic purpura might be due to specific antibodies active against platelets and also probably against the vascular endothelium This idea lay dormant, however until 1949 when Evans and Duane found that a number of patients with acquired haemolytic anaemia due to red cell agglutinins also had thrombocytopenia and later (Evans et al 1951) showed that platelet agglutinins could be demonstrated in some cases of idiopathic thrombocytopenia Supporting evidence was provided by studies of the survival of transfused platelets which were found to be destroyed abnormally rapidly in the circulation of idiopathic thrombocytopenic patients though persisting for a normal period of 4-5 days in cases of thrombocytopenia due to hypoplasia of the marrow (Stefanini et al 1952 Hirsch and Gardner 1951) and by the observation of Harrington et al (1951, 1953) that transfusion of blood from cases of idiopathic

of the purpuric type since there is often spontaneous bleeding from the mucous membranes and from the internal surfaces of the body and a lack of the massive tissue haemorrhages so characteristic of the states due to defective coagulation It must be emphasized however that though the description purpuric has been used it is not applied in its strict sense of spontaneous haemorrhage into the skin Petechiae and ecchymoses may occur in some of these non-thrombocytopenic states but they are not a characteristic feature In some of these cases there is direct evidence that a capillary defect is the cause of the abnormal bleeding as in the specific condition of hereditary haemorrhagic telangiectasia. In the majority there is not direct evidence of a vascular abnormality yet the patient suffers severely from haemorrhage despite a normal platelet count, and apparently efficient blood coagulation It has therefore been postulated by many authorities that there exists in these patients a qualitative or functional defect of the platelets so that they are suffering in effect from a functional thrombocytopenia

The literature dealing with these cases is most confused partly because a number of different names have been applied to the same clinical condition and partly because the investigations carried out were sometimes inadequate to justify conclusions on the cause of the abnormal bleeding There is sufficient evidence however to show that they can be divided into two main groups though there may be some overlapping between them One group consists of cases of a hereditary haemorrhagic state in which the only constant abnormal finding is a prolonged bleeding time and in which the platelets appear to be morphologically and functionally normal this condition will be referred to as von Willebrand s disease The other group is more heterogeneous it includes all cases of non-thrombocytopenic purpura in which functional deficiency of the platelets can be inferred from their abnormal morphology or their failure to clump or form pseudopodia, or from a deficiency of clot retraction or prothrombin consumption or the failure of the platelets to participate in thromboplastin generation Individual cases in this latter group differ considerably in their clinical and laboratory manifestations in some the haemorrhagic state is hereditary in others it is sporadic in many it is acquired and may be secondary to leukaemia, polycythemia or toxic damage The general term thromboasthenia is used here to cover this group

indicate a specific antibody reaction they may only indicate the presence in the test system of one or more non-specific factors *including* derivatives of the coagulation system or physical factors which can promote agglutination The present situation though interesting and promising a considerable clarification must still be regarded with caution The subject is well reviewed by Ackroyd (1955)

As regards the effects of treatment an immunological basis for many cases of 'idiopathic' thrombocytopenic purpura would if established, allow a better understanding The beneficial effect of splenectomy as in certain cases of haemolytic anaemia might be ascribed to a reduction in the rate of destruction of platelets damaged by antibodies, and in the rate of production of the antibodies themselves It is significant however that platelet agglutinins could still be demonstrated in the blood of some patients after splenectomy had effected a clinical cure (Harrington et al 1951 Stefanini et al 1953) though in others autoagglutinins disappeared after the operation The action of A C T H and cortisone as in the haemolytic anaemias is obscure They may reduce the formation of antibodies but in many cases clinical improvement is not accompanied by a rise in the platelet count and it must be postulated that these substances protect the vascular endothelium or increase its haemostatic function The effect of platelet transfusion is interesting clinical improvement may last for two or more days though the transfused platelets may disappear in a few hours (Stefanini et al 1952) Clinical improvement is not therefore due directly to the presence of the platelets in the circulation, it might be due to haemostatic factors (such as 5-hydroxy-tryptamine) released by the platelets during their disintegration after transfusion or it might be due to a reduction of the circulating platelet (and endothelium) antibody caused by its absorbtion by the transfused platelets

NON-THROMBOCYTOPENIC PURPURA HAEMORRHAGICA

When the published descriptions of the haemorrhagic states are studied it is found that in a proportion of cases the abnormal bleeding cannot be attributed either to defective coagulation or to thrombocytopenia In most of these the haemorrhagic tendency is

used also gave similarly abnormal results in female carriers of haemophilia a finding which is contrary to that of other workers (Merskey and Macfarlane 1951) More specific examination of the thromboplastic activity of the platelets in von Willebrand s disease has not yet been carried out in more than a few cases Soulier and Larrieu (1954) report it to be normal in one case examined and in our own experience it was normal in the three cases investigated in this way Braunsteiner (1955) in an extensive investigation of 24 cases of thrombopathy describes 7 cases in which the bleeding time was prolonged but clot retraction prothrombin consumption platelet thromboplastic activity clumping and pseudopodia-formation were normal The son of one of these patients who had a similar clinical condition showed apparently some diminution of prothrombin consumption and lack of platelet clumping suggesting that findings may vary between members of the same family or from time to time in the same individual

The Features of von Willebrand s Disease

Clinical Manifestations

The tendency to bleed appears in childhood It takes the form of bruising epistaxis bleeding from the gums gastro-intestinal bleeding and prolonged bleeding from injuries and operation sites Menorrhagia may be the most serious symptom in women Petechiae and ecchymoses may occur but such purpura is not very common Deep tissue haemorrhages are rare but in about 8 per cent of reported cases there seems to have been some joint involvement The sexes are equally affected

Inheritance

There is a positive family history in over 80 per cent of reported cases In most families inheritance is of the simple dominant type the affected members passing the condition to their children Some authors consider that in some families sex-linkage occurs

Laboratory Findings

The essential abnormal findings is the prolonged bleeding time The degree of prolongation varies from one case to another In most cases the prolongation is considerable and may extend to many hours if the patience of the subject and the investigator allows The tourniquet test is variable being positive in about half the cases

VON WILLEBRAND'S DISEASE

In 1931 von Willebrand gave the first clear descriptions of a haemorrhagic diathesis inherited as a Mendelian dominant affecting both sexes and characterized by a prolonged bleeding time normal clotting time and normal platelet count In the one case in which it was investigated, the clot retraction was normal von Willebrand and Jurgens (1933) sought to show by means of a special apparatus that the agglutination of the platelets was deficient in this condition Though the results of this experiment are difficult to interpret it has led to a tendency to regard von Willebrand s cases and similar cases described later by other authors as examples of thromboasthenia a condition which had been described by Glanzmann in 1918 and which was essentially a haemorrhagic state characterized by deficient clot retraction and abnormal platelet morphology

Macfarlane (1941) described five cases resembling those of von Willebrand and found that no platelet abnormality could be demonstrated and clot retraction was normal but that the capillaries of the nail folds were abnormal in shape and failed to contract after puncture He suggested that the bleeding in these patients was caused by an inherited capillary defect not by a functional platelet deficiency and pointed out that in a number of probably similar cases described in the literature under a variety of titles clot retraction was usually reported to be normal

By 1946 Estren Medal and Dameshek were able to review 62 cases from the literature adding 11 of their own They applied von Willebrand s original term pseudohaemophilia to the condition, though there is little clinical genetic or pathological resemblance to true haemophilia In 1950 Revol Favre-Gilly and Ollagnier reviewed the 91 cases then available 11 new cases were added by Soulier and Larrieu (1954) and the investigation of 21 affected members of one family of von Willebrand s disease were reported by MacFarlane and Simpkiss (1954) In all these reviews the apparent normality of platelet function was stressed We have found that in 8 cases examined the prothrombin consumption test gave normal results Similar results have been obtained by Lelong and Soulier (1950) Cazal and Izarn (1950a) Andre (1952) and Soulier and Larrieu (1954) in a total of 20 cases Jurgens and Ferlin (1950) reported a reduced prothrombin consumption in 9 cases of constitutional thrombopathy which they regard as synonymous with von Willebrand s disease but it may be significant that the technique

TABLE 32

LABORATORY TESTS ON PATIENTS WITH VON WILLEBRAND'S DISEASE

Case	*Inheritance*	*Bleeding Time*	*Tourniquet Test*	*Platelets c mm*	*Clotting Time (minutes)*	*Prothrombin Consumption Index*	*Level of A H G*
Case 37 (Mother)	Dominant	15+	+	* 400 000	$5\frac{1}{2}$	20	8 6
Case 38 (Son)		15+	+	Normal Normal	—	—	1 6
Case 39 (Female)	None	15+	negative	* 500 000	$8\frac{1}{4}$	25	0
Case 40 (Female)	Dominant	15+	—	560 000	$6\frac{1}{2}$	Less than 13	12
Case 41 (Grandmother)	Dominant	15+	+	196 000	$5\frac{3}{4}$	15	9
Case 42 (Mother)		15+	—	* 219 000	7	17	26
Case 43 (Son)		15+	—	257 000	$5\frac{3}{4}$	15	26
Case 44 (Father)	Dominant	3 min.		211 000	$5\frac{1}{2}$	Less than 5	33
Case 45 (Daughter)		15+		237 000	$9\frac{1}{2}$	Less than 10	26

* The Thromboplastin generation test carried out using the patient's platelets to replace normal, gave normal results

The platelet count is usually within normal limits but may be raised in a few cases Most observers agree that there is no morphological abnormality and that platelet clumping occurs normally on glass surfaces (Estren et al 1946, Revol et al 1950, Soulier and Larrieu 1954) The blood coagulation time was normal in 96 per cent of the cases reported and was slightly prolonged in the remainder Prothrombin times where estimated were normal and prothrombin consumption has been reported to be normal by the majority of workers who have investigated it as already mentioned with the exception of Jurgens and Ferlin (1950) Clot retraction was reported to be normal in 69 of 73 cases described in the literature A recent and remarkable finding is that the blood level of antihaemophilic globulin may be significantly reduced in cases of von Willebrand s disease Larrieu and Soulier (1953) and Alexander and Goldstein (1953) describe 2 cases in which A H G deficiency was established by the failure of the patient's blood to correct the clotting defect of haemophilic blood We have assayed the blood A H G activity in 8 cases of von Willebrand s disease, and found that it ranged from 33 per cent of normal to 1 6 per cent (see Table 32) The facts that prothrombin consumption was within normal limits despite these reductions of A H G and that the clinical symptoms bore no resemblance to those of haemophilia are at present inexplicable

Capillary morphology and function were found to be abnormal by Macfarlane (1941) Levy (1947) Cazal and Izan (1950a) Perkins (1946) and O Brien (1950) in a total of 17 cases O'Brien (1950) has pointed out that both in hereditary haemorrhagic telangietasia and in von Willebrand s disease there is a capillary defect giving rise to haemorrhage inherited in a similar way and that both conditions may occur in the same families He has suggested the name diffuse capillary telangietasia for von Willebrand s disease It is of interest that blood levels of A H G were normal in 2 cases of telangiectasia examined by us

Treatment

Only local and palliative treatment is of any value in this condition Various substances supposed to influence capillaries have been tried such as Vitamins P and C, Rutin adrenochrome and A C T H but no significant improvement has been obtained Splenectomy is both useless and dangerous An artificial menopause may be the only effective method for dealing with severe menorrhagia

of the platelets Bernard and Soulier (1948) and Favre-Gilly and Dameshek (1950) describe cases of a haemorrhagic diathesis in children in which a prolonged bleeding time was associated with normal clot retraction but deficient prothrombin consumption The main feature in these cases was the abnormal size of the platelets which were up to 8μ in diameter In the case described by Favre-Gilly and Dameshek (1950) transfusion of normal platelets restored the prothrombin consumption and bleeding time tests to normal and the disappearance from the circulation of the normal platelets (recognizable because of their morphological difference from the patient s own platelets) was accompanied by a return of abnormal prothrombin consumption and some time later of a lengthened bleeding time Alexander and Landwehr (1949b) investigated a case of acquired haemorrhagic diathesis in which the morphologically abnormal platelets were shown to be incapable of promoting prothrombin consumption when added to normal platelet free plasma Other cases of morphological platelet abnormality are described by Revol (1945) Guichard and Revol (1949) Sameck (1930) Manci (1947) Panoff (1948) and Quatrin (1946) and these and others are reviewed by Clavel (1950) In general they are characterized by abnormal platelet morphology absence of clumping on glass surfaces and in most cases reduced clot retraction In a few instances there was a family history suggesting a possibly dominant inheritance in most the condition appeared to be constitutional but sporadic or secondary to some blood disease

The most extensive investigation of cases of qualitative platelet abnormalities has been carried out by Braunsteiner (1955) who also gives a full review of the literature In a study of 24 patients in whom the bleeding time was prolonged but the platelet count normal he found 16 cases of demonstrable platelet abnormality He divided these into two groups those with thrombopathy and those with thromboasthenia In the cases of thrombopathy the main abnormality was a deficiency of the coagulation function of the platelets demonstrated by deficient prothrombin consumption and decreased thromboplastin forming ability Other platelet functions such as clot retraction clumping and pseudopodia formation were normal In the cases of thromboasthenia the clotting function of the platelets indicated by prothrombin consumption and thromboplastin generation was normal but clot retraction clumping and pseudopodia formation were deficient He concludes that the

Pathogenesis

It seems probable that von Willebrand's disease is due to an inherited abnormality of the capillaries of the skin and mucous membranes. The absence of deep tissue bleeding and of serious haemorrhage following operations which do not involve large surface areas, suggests that the vessels of deeper tissues are haemostatically normal. There is no satisfactory evidence that the platelets are abnormal, but the study of platelet function is still in its infancy and apparently primary vascular defects may prove to be the results of abnormalities of some platelet action not yet recognized. The reduction of A H G observed in a number of cases of von Willebrand's disease is puzzling from more than one point of view. It is strange that reduction of this order should have relatively little effect on clotting function as usually measured and should not produce the clinical manifestations of haemophilia. The administration of A H G to 2 cases did not, in our experience, improve the haemorrhagic tendencies nor shorten the bleeding time. The mechanism of the A H G deficiency is also obscure: it is possible that widespread capillary defects and superficial bleeding might cause an increased consumption of A H G. Brinkhous and Penick (1954) have observed reduction of A H G in the blood of animals after damage to the skin by cold injury.

FUNCTIONAL PLATELET DEFICIENCY (THROMBOASTHENIA)

It seems that Glanzmann (1918) was the first to suggest that a functional platelet defect might be a cause of abnormal bleeding. He described under the name 'thromboasthenia' cases of excessive haemorrhage in which the bleeding time, platelet count and clotting time were normal, but in which clot retraction was deficient and the platelet morphology abnormal. This picture became confused, and his observations discredited, when cases labelled 'thromboasthenia' were described by later observers which were in reality examples of von Willebrand's disease having normal clot retraction and platelet morphology and a prolonged bleeding time.

Though it is probable that some of Glanzmann's patients were actually suffering from a mild form of thrombocytopenic purpura (Budtz-Olsen 1951) it is also probable that others actually had thromboasthenia, though this point cannot now be established.

In recent years a number of cases have been reported in which investigations have been sufficient to establish functional deficiency

between platelet number and tendency to bleed It is suggested that since platelets and capillary endothelium are known to be antigenically similar that antibodies which have been demonstrated in some cases may damage both capillaries and platelets

Thrombocytopenia and purpura may be caused in susceptible individuals by sensitization to drugs such as sedormid (Ackroyd 1949a and b 1951) In these cases it is probable that the drug forms with the platelets an antigenic complex to which an antibody is formed In the presence of the antibody sensitized platelets are agglutinated and lysed In these patients there is evidence that, in the presence of the antigen the circulating antibody may damage the capillaries directly

Thrombocytopenia may also occur in acute infections as a result of poisoning with a variety of substances and when the bone marrow is aplastic or replaced by leukaemic or other tissue

Thrombocytopenia is not usually associated with any lengthening of the coagulation time but in the majority of cases a marked reduction in prothrombin consumption is present The bleeding time is usually prolonged and the tourniquet test positive

A haemorrhagic disease inherited as a Mendelian dominant has been called von Willebrand s disease thromboasthenia Glanzmann s disease thromboasthenia constitutional thrombopathy hemogenia etc The term von Willebrand s disease is preferred The disease is characterized by the familial incidence of a haemorrhagic disease dating from childhood The capillaries are abnormal in morphology and the bleeding time prolonged The platelets are normal in number and the coagulation system may show a deficiency of A H G

A small number of cases has been described in which the morphology of the platelets is abnormal and in which deficient clot retraction, or reduced prothrombin consumption or reduced thromboplastin forming capacity despite a normal number of platelets suggests that there was a deficiency of platelet function The patients concerned suffered from abnormal haemorrhage No constant aetiological factor was apparent

pathogenesis of the bleeding in these states may be related to a clotting defect produced by thrombopathy', and to a delay in forming adhesive platelet clumps in 'thromboasthenia' Van Creveld and Paulssen (1953) had also stressed the importance of a deficiency of the platelet thromboplastic factor (Factor 3) in producing a haemorrhagic diathesis which they have referred to as 'thrombopathia haemophilica

It should be emphasized that the division of these cases into groups of this sort may be arbitrary, and that von Willebrand s disease, thrombopathy and thromboasthenia may represent different phases of the same condition or artificial selections of a naturally heterogeneous group in which many variants of hereditary or acquired abnormalities of platelets and blood vessels may be combined in different ways

HAEMORRHAGIC THROMBOCYTHAEMIA

There have been a number of descriptions of a haemorrhagic state similar to that of thromboasthenia but associated with a greatly increased platelet count Mortensen (1948) describes a case of *polycythaemia* with a haemorrhagic diathesis a prolonged bleeding time and a platelet count of 6 million per c mm Holst (1948) describes a similar case and quotes ten others from the literature The condition seems to be a secondary one associated with polycythaemia or leukaemia and the mechanism of the bleeding tendency has not been determined

SUMMARY TO CHAPTER XVI

Following a reaction with antihaemophilic globulin Christmas factor and $CaCl_2$ platelets provide an important component of plasma thromboplastin Since the concentration of plasma thromboplastin influences thrombin formation reduction in platelets has a marked effect on the consumption of prothrombin during coagulation Reduction in platelets reduces the amount of thromboplastin formed but has no great effect on the speed of its formation and thus moderate reduction in platelets does not lengthen the clotting time

In essential thrombocytopenia (Werlhof s disease) platelets are reduced in number and capillary bleeding occurs Although the two features are often associated there is no absolute correlation

is due to a similar mechanism (Hoigné 1951) The condition is complicated by the fact that agglutination of the platelets occurs in anaphylaxis so that they may be very considerably reduced in number (Quick, Ota and Baronofsky 1946) The anticoagulant effect of peptone when given by intravenous injection a phenomenon which was intensively investigated by early observers is probably related to the effect of heparin Like heparin peptone is about fifteen times more effective as an anticoagulant when given in vivo than when added to the blood in vitro (Camus and Gley 1896) If the peptone is added to the blood which has been withdrawn from the body using paraffined apparatus it is about ten times more effective than if added to blood which has been in contact with glass (Pickering 1928) It would seem therefore that both peptone and heparin inhibit a change which occurs in the blood possibly platelet lysis or some other factor concerned in thromboplastin generation which occurs on contact with foreign surfaces Intravenous injection of peptone probably causes an increase in blood heparin (Howell 1925) and the platelet deficiency is as marked as it is in anaphylaxis

Allen and Jacobsen (1947) have reported that exposure to ionizing radiation caused a rise in blood heparin sufficient to produce partial or complete incoagulability This observation was extended and confirmed by Allen et al (1948) who found that the lengthening of the coagulation time that occurred in dogs exposed to X-rays could be corrected by the addition of toluidine blue or protamine Copley (1948) has shown that heparin may actually induce platelet agglutination and petechial haemorrhages due to platelet emboli and suggests that the thrombocytopenia observed in subjects exposed to atomic bomb radiation is due to the increase of blood heparin These observations are clearly of considerable interest in view of the increasing hazards of the atomic age but Cronkite (1950) believed that the haemorrhagic syndrome of 'acute ionizing radiation illness produced in goats and pigs by exposure to the effects of the atomic bomb exploded at Bikini was predominantly due to vascular lesions and thrombocytopenia and that in only a few cases was it due to a heparin-like substance in the blood

There have been a number of reports of spontaneous haemorrhagic states due to a clotting defect in which a heparin-like anticoagulant appeared to be wholly or partly responsible Castex (1946) and Castex and Pavlovsky (1947) reported the occurrence of anticoagulants of the heparin type in human cases Conley Hartmann

CHAPTER XVII

NATURALLY OCCURRING ANTICOAGULANTS

In their *preoccupation with the factors concerned in coagulation* most workers have under-emphasized the importance of the normal maintenance of blood fluidity But to the individual the liquid state of his blood in the vascular system is more important than its ability to coagulate outside it and more people die of thrombosis than of hypocoagulability Normally the blood has an inherent stability within the body mainly achieved by the unbroken continuity of the vascular endothelium which provides no foreign surface to stimulate the formation of thromboplastic activity Though disease may destroy the integrity of this vascular surface it is only in extreme cases that it leads to more than local intravascular coagulation The extensive damage to tissues and vessels that occurs as a result of surgical or accidental trauma must lead to absorption of thromboplastin and thrombin but in most cases only that amount of intravascular coagulation occurs which is required to effect haemostasis This normal reluctance of the mass of circulating blood to clot even after contact with a foreign surface or in the presence of small concentrations of thrombin or thromboplastin is due to an anticoagulant system which is an important safeguard against thrombosis The known factors in this system are the antithrombin of the plasma and heparin and there is also some evidence suggesting the existence of an antithromboplastin The nature and mode of action of these factors is described in previous chapters In certain conditions an abnormal increase in the anticoagulant activity of the blood may occur with consequent impairment of normal clotting efficiency

THE INHIBITION OF THROMBIN

An inhibition of thrombin is a relatively unusual cause of defective coagulation in man A considerable rise in the blood heparin has been shown to occur in anaphylactic shock in dogs by Jaques and Waters (1941) and it is probable that the decreased coagulability of the blood which has been described in human cases of anaphylaxis

in the blood of haemophilics following repeated transfusions is the result of immunization by antihaemophilic globulin which may in such subjects act as an antigen This immunization is a very serious complication in the treatment of haemophilia but in our experience it does not occur very frequently The first definite observation of a deleterious effect in haemophilia following the administration of antihaemophilic factor seems to have been made by Pohle and Taylor (1937) They found that administration of their preparation caused a refractory phase during which the patient was resistant to further administrations In a later communication Pohle and Taylor (1938) seem to have obviated this difficulty by changing the method of preparation and the nature of this particular refractory phase does not seem to have been established Munro and Jones (1943) described the detrimental effect of frequent transfusions in a case of haemophilia The patient who had previously responded well to transfusion became resistant and failed to show either a reduction in the clotting time or an improvement in his clinical state on further transfusions Munro (1946) investigated the patient and found that a thermostable anticoagulant which was not heparin could be detected in the blood Munro and Munro (1946) carried out electrophoresis of the plasma and found that the anticoagulant was associated with the gamma globulin suggesting that it might be an antibody Craddock and Lawrence (1947) described anticoagulants which developed in two cases of haemophilia following the injection of Cohn s fraction I These anticoagulants also present in the gamma globulin fraction of the plasma gave specific precipitin reactions with antihaemophilic globulin and inhibited its ability to accelerate the coagulation of haemophilic blood It seems to be established therefore that injection of antihaemophilic globulin in these two patients resulted in the formation of a definite antibody to this factor Frommeyer Epstein and Taylor (1950) record that of sixteen haemophilic patients who were treated with plasma fractions five developed anticoagulants and precipitin reactions against antihaemophilic globulin were observed They found that disappearance of these anticoagulants occurred if the patients were transfused with massive amounts of normal blood and that the refractory state could be abolished at any rate temporarily by this treatment Other instances are reported by Lawrence and Johnson (1946) Soulier and Burstein (1948) Conley et al (1948) Trank, Soulier and Blatrix (1949) and Singer et al

and Morse (1949b) described 8 cases in which a clotting defect was associated with circulating anticoagulants but only one of these showed the presence of a heparin-like substance Rosenthal (1949) has found a coagulation defect in 14 out of 28 patients with chronic leukaemia associated with a decreased heparin tolerance suggesting an increased heparin content of the blood and it has been claimed by Allen et al (1947) that the haemorrhagic manifestations in thrombocytopenic purpura can be alleviated by treatment with protamine also suggesting an increased blood heparin in these cases Koller et al (1950) found evidence of an increased blood heparin in a case of purpura fulminans Crisalli and Cotellessa (1950) described a 10-year-old boy with a greatly prolonged coagulation time and symptoms resembling haemophilia in which the clotting defect was corrected by toluidine blue and protamine Jorpes (1946) implies that the haemorrhagic condition in urticaria pigmentosa in which mast cells rich in heparin accumulate about the capillaries of the skin is due to heparinaemia Meneghini (1951) has found a correlation between the mast cells in the marrow and the heparin activity of the blood in a number of cases of leukaemia liver disease, purpura and in patients after irradiation

INHIBITION OF INTRINSIC THROMBOPLASTIN

It is now becoming apparent that in a number of different cases diagnosed as haemophilia or pseudo-haemophilia bleeding was due to the development of anticoagulants apparently active against thromboplastin or delaying its normal generation in the plasma It seems that there are at least three ways in which such anticoagulants can appear The best defined group consists of genuine haemophilic patients *who have become resistant to blood transfusion* and in whom the existence of a factor inhibiting antihaemophilic globulin can be demonstrated The second group consists of women who soon after childbirth have developed a haemorrhagic diathesis which is apparently due to the development of an anticoagulant very similar to that observed in the first group The third group includes patients with circulating anticoagulants of unknown cause Data from some published cases is summarized in Table 33 pp 304-5

ANTICOAGULANTS IN HAEMOPHILIC PATIENTS

It is very probable that the acquired anticoagulant which appears

Frommeyer et al (1950)	5 cases ♂	Haemophilia				Not controlled by transfusion. Precipitin to A.H.G. 1/128–1/2048
Singer et al 1 (1950)	67 ♂	Cardiac infarction	105 minutes	10	25	Not corrected by A.H.G. Corrected by ground platelets. No precipitins demonstrated
2	4 ♂	Haemophilia	90 minutes	30	60–120	
Van Creveld et al. (1951)	28 ♂	Haemophilia	4 hours +	10	35 minutes	Neutralized A.H.G.
Joules and Macfarlane (1938)	56 ♀		48 minutes			Did not shorten the clotting time of haemophilic blood
Madison and Quick (1945)	30 ♀	Pregnancy	17 minutes			
Chargaff and West (1946)	33 ♀	Pregnancy	80–100 minutes			
Fantl and Nance (1946 b)	39 ♀	Pregnancy	900–2200 seconds	67	1200 seconds	
Hewlett and 1 Hadan (1949)	40 ♀	Pregnancy	1 hour & 56 minutes			Did not shorten the clotting time of haemophilic blood
2	33 ♀	Pregnancy	2 hours & 45 minutes			
Heinle et al (1949)	♀	Pregnancy	Long		Prolonged	Abnormal constituent in the albumin fraction. No figures given
Dreskin and Rosenthal (1950)	30 ♀	Pregnancy	25–90 minutes	33	16	Neutralized A.H.G.
Biggs and Macfarlane	35 ♀	Pregnancy	26–50 minutes	For details see text		Neutralized A.H.G.

TABLE 33

PATIENTS WITH CIRCULATING ANTICOAGULANTS

Author	*Age and sex*	*Associated cond tion*	*Whole blood or plasma clotting time*	*Effect on Clotting Time of normal blood or plasma*		*Notes*
				Per cent of patient s plasma	*Clotting time of mixture*	
Lozner et al (1940)	61 ♂	Lymph node tuberculosis	68–90 minutes	10	42 minutes	
Lawrence and Johnson (1946)	44 ♂	Haemophilia	60–115 minutes	30	15	Inhibitor in gamma globulin Pre cipitin to A.H G 1/320
Craddock and Lawrence (1947)	21 ♂	Haemophilia	4–6 hours	40	15 minutes	Inhibitor in gamma globulin Pre-cipitin to A H G 1/160
Munro (1946)	♂	Haemophilia	150 minutes	33	50	Inhibitor in gamma globulin fraction
Soulier and Burstein (1948)	21 ♂	Haemophilia	360 minutes	10	26–48	
Conley et al 1 (1948) 2	67 ♂ 30 ♂	Chronic nephritis	68 minutes 60 minutes	10 10	39 39	
3	38 ♂	Haemophilia	5 hours	10	5 minutes	
Dieter et al (1949)	68 ♂	Pemphigus	90–2 0 minutes	10	23	
Tzank et al (1949)	67 ♂	Dermatitis	2 hours	10	3½ minut s (N 1 45)	Not controlled by t an fu on

therefore that the anticoagulants developing in these women may be of the same type as those found in haemophilics who become resistant to the administration of blood or plasma fractions It is tempting to suppose that some foetal-maternal immunization process may have been responsible Dreskin and Rosenthal (1950) have suggested that immunization by placental fragments may cause the development of an antithromboplastic immune body

As a result of these observations we were able to study a similar case in detail

Case Report

The patient was a woman aged thirty five who eight months after the birth of her second child developed a haemorrhagic diathesis sufficiently severe to necessitate two blood transfusions On investigation of the haemostatic defect the following preliminary observations were made

Whole blood clotting time		
(Lee and White *1913*)		*26–50 minutes*
Bleeding time		2 minutes 5 seconds
Tourniquet test		Weak positive
Platelets		173 000–350 000 c mm.
One-stage prothrombin test		
(Quick 1935)		65 100 per cent
Prothrombin consumption index		
(Merskey 1950)		120 per cent
Plasma protein total		7 gms per cent
Albumin	5 gms per cent	
Globulin	1 8 gms per cent	
Fibrinogen	0 2 gms per cent	

From these preliminary tests the haemorrhagic diathesis seemed to be explained by the clotting defect. Deficiency in Factor V Factor VII and prothrombin could be excluded because the one-stage prothrombin test gave normal results and the thrombin fibrinogen reaction was found to be normal No anticoagulant of the heparin type could be demonstrated the addition of toluidine blue did not shorten the clotting time The antithrombin activity of the patient s blood measured by determining the speed of thrombin neutralization by the patient s plasma was normal

TABLE 34

THE REACTION OF THE PATIENT S PLASMA TO INCREASING DILUTIONS OF BRAIN THROMBOPLASTIN COMPARED TO HAEMOPHILIC AND NORMAL PLASMA

To 0 1 ml of plasma was added 0 1 ml. of brain emulsion in saline and 0 1 ml of M/40 CaCl The clotting time in seconds from the addition of CaCl was recorded

Plasma sample	*Dilution of brain thromboplastin*				
	1/10	1/100	1/1000	1/10 000	*No brain*
Normal	28	50	82	115	149
Patient	29	77	225	570	1200
Normal	17	33	66	119	183
Haemophilic	22	44	68	200	1140

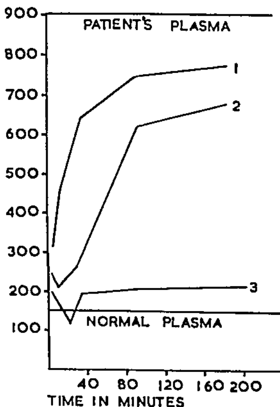
(1950) There are minor differences in the observed nature of the anticoagulant *in some of these cases* for instance Soulier and Burstein found that frozen and thawed platelets seemed capable of overcoming the anticoagulant activity in the plasma of their patient which might support Quick s (1951a) contention that anticoagulants of this type act by inhibiting platelet function It is true that not in all cases was it possible to demonstrate precipitins against antihaemophilic globulin but their unequivocal occurrence in other cases, together with the demonstration that the action of antihaemophilic globulin is inhibited strongly suggests that it is this factor against which the anticoagulant is operative

This rather gloomy view of the prognosis for transfused haemophilic patients is not supported by our own experience We have now seen patients with haemophilia from over 100 families Most of them have had numerous transfusions and in only two instances has a demonstrable circulating anticoagulant developed Resistance to transfusion is not a good criterion for the presence of a circulating anticoagulant all haemophilic patients are resistant to transfusion in that very large amounts of blood may be required to achieve a haemostatic level of circulating antihaemophilic globulin

PSEUDO-HAEMOPHILIA IN FEMALES FOLLOWING PREGNANCY

The second group of cases consists of women *in whom a haemorrhagic* diathesis almost indistinguishable from haemophilia has arisen usually quite suddenly in adult life In the majority of the instances reported the bleeding tendency has occurred within a few weeks or months of the birth of a child The laboratory findings resemble those of haemophilia very closely there being a prolonged coagulation time a normal one-stage prothrombin time normal platelet count and no demonstrable increase in antithrombin Madison and Quick (1945) Lovemann (1945) Chargaff and West (1946) Fantl and Nance (1946b) Hewlett and Hadan (1949) Heinle et al (1949) and Dreskin and Rosenthal (1950) have described *a total of eight patients all being women who developed a haemorrhagic* diathesis characterized by the presence of an anticoagulant within a short time of delivery In most cases the patient s blood inhibited the coagulation of normal blood and in some (Hewlett and Hadan 1949 Dreskin and Rosenthal 1950) there was a failure of the patient s blood to correct the clotting defect in haemophilia and inhibition of the normal antihaemophilic factor It seems likely

A series of mixtures of the patient s plasma and normal plasma were then made and the clotting times on recalcification were recorded From Table 36 it will be seen that though the addition of normal plasma shortened the clotting time of the patient s plasma the effect was relatively less than occurs in mixtures of normal and haemophilic plasma When various normal plasma fractions were added to the p tient s plasma it was found that the substance which was responsible for the shortening was present in the fibrinogen fraction which also contains the antihaemophil c globul n It will also be seen that though the clotting time of the pat ent s plasma is shortened by normal plasma, the clotting time of normal plasma is length ened by the patient s plasma One part of the abnormal plasma in nine of normal plasma lengthened the latter s clotting time from 149 to 217 seconds It appears therefore that the patient s pl sma contains an inhibitor It was found that this inhibitor has a progressive



F g 47 Mixtures of normal and pathological plasma were incubated tog ther and at intervals the calcium lotting tim test was carried out on samples (1) 9 parts of patient's plasma 1 part of normal plasma (2) 9 parts of normal plasma 1 p rt of p tient s plasma (3) 1 p rt of normal plasma, 9 parts of haemophilic plasma The calcium clotting time of the normal and the patient s plasma are shown for comparison

Samples of the patient s plasma and normal plasma were recalcified in the presence of increasing dilutions of human brain thromboplastin and the clotting times were recorded From Table 26 it will be seen that the clotting times of the patient s plasma with weak suspensions of thromboplastin were longer than those of the normal plasma a discrepancy already observed in the reaction of haemophilic plasma to dilute brain thromboplastin (Biggs and Macfarlane 1951) and also illustrated in Table 34

To determine whether or not the patient s plasma had the ability to destroy thromboplastin a sample of plasma was incubated with brain thromboplastin for one hour and at intervals the residual thromboplastic activity of samples was tested. From Table 35 it will be seen that there was no deterioration of thromboplastic activity on incubation of brain emulsion with the patient s plasma From these experiments it can be concluded that there is an abnormal reaction with weak brain but no destruction of its activity These results are similar to those obtained with haemophilic plasma

TABLE 35

THE ABILITY OF THE PATIENT S PLASMA TO INACTIVATE BRAIN THROMBOPLASTIN

0 5 ml of plasma was incubated with 0 5 ml of brain emulsion and at intervals 0 1 ml sub-samples were added to 0 1 ml of normal plasma and the mixture recalcified with 0 1 ml of M/40 $CaCl_2$ The clotting times of the sub-samples in seconds were recorded

Plasma sample	*The time for which the thromboplastin plasma mixtures were incubated*				
	4 min	*8 min*	*16 min*	*30 min*	*60 min*
Normal	76	76	80	83	1-5
Patient	73	73	77	65	70

TABLE 36

THE ABILITY OF NORMAL PLASMA TO SHORTEN THE CLOTTING TIME ON RECALCIFICATION OF THE PATIENT S PLASMA AND OF HAEMOPHILIC PLASMA

The patient s and normal plasma were mixed in the proportions shown 0 1 ml *of plasma was mixed with 0 1 ml of 0 85 per cent saline and 0 1 ml* of M/40 $CaCl_2$ was added The clotting times of the mixtures in seconds were recorded The experiment was repeated with similar mixtures of normal and haemophilic plasma

	Proportions of normal and patient s plasma mixed together							
	Normal Abnormal	1 0	0 9 0 1	0 7 0 3	0 5 0 5	0 3 0 7	0 1 0 9	0 1
Mixture of plasmas tested	Normal and patient	149	217	273	320	380	458	1200
	Normal and haemophilic	205	—	255	240	240	295	1620

plasma. In an attempt to locate this inhibitor fractions were prepared from normal and the patient s plasma by Dr R. A Keckwick using the ether precipitation method (Keckwick and Mackay 1949) These fractions were

1 A gamma globulin fraction
2 A beta globulin fraction which also contained some alpha globulin.
3 An albumin fraction which also contained some alpha globulin.
4 A fibrinogen fraction which contained the antihaemophilic globulin

From tests that were carried out it appeared that the inhibitor responsible for the clotting defect was present in the gamma globulin fraction

When the patient s plasma was mixed with haemophilic plasma there was no shortening of the clotting time on recalcification (Table 37) From this it appears that either the patient s pl sma lacks the antihaemophilic glob lin or the inhib tor masks its action The fibrinogen fraction isolated from the patient, which was itself only very sl ghtly inhibitory did not shorten the clotting time of haemophilic plasma and therefore did not contain antihaemophilic globulin. It m y be concluded therefore that the patient's plasma lacked the antihaemophilic

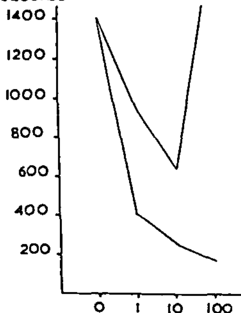


Fig 48 Mixtures of part of normal and part of the p tient s gamma globulin fracti n were incubated together for two hours At the end of this time the mixture was diluted 1/10 and 1/100 with 0 85 per cent saline 0 1 ml. of the dilutions was added to 0.1 ml. of haemophilic plasma and the mixtures recalcified. The results of this experiment re shown in the upper curve Similar dilutions of untreated normal plasma were tested for comparison (lower curve) It will be seen that the p tient s gamma globulin fraction reduced the anti haemophilic effect of normal plasma

activity because when normal plasma was incubated with a proportion of the patient s plasma and samples were recalcified at intervals the clotting time of the mixture was lengthened as the incubation time progressed This effect is illustrated in Fig 47 where it will be seen also that haemophilic plasma has no such progressive inhibitory effect on the clotting of normal

TABLE 37

THE CLOTTING TIMES ON RECALCIFICATION OF MIXTURES OF HAEMOPHILIC AND THE PATIENT S PLASMA

Haemophilic and the patient s plasma were mixed in the proportions shown To 0 1 ml of the plasma mixture was added 0 1 ml of 0 85 per cent saline and 0 1 ml of M/40 $CaCl_2$ The clotting times were recorded in seconds

	Proportions of patient s and haemophilic plasma mixed together							
Haemophilic Patient	1 0	0 95 0 05	0 9 0 1	0 7 0 3	0 5 0 5	0 3 0 7	0 1 0 9	0 1
	1620	16 0	1380	1200	1260	1260	1270	1330

TABLE 38

THE EFFECT OF INCUBATING THE ANTIHAEMOPHILIC FACTOR (PREPARED BY PRECIPITATION OF NORMAL PLASMA AT 33 PER CENT SATURATION WITH AMMONIUM SULPHATE) WITH HAEMOPHILIC AND THE PATIENT S PLASMA

The fibrinogen was incubated for 10 minutes at 37 C with an equal volume of saline haemophilic plasma or the patient s plasma At the end of this time 0 1 ml of each of the mixtures was added to 0 1 ml of haemophilic or patient s plasma and recalcified with 0 1 ml of M/40 $CaCl_2$ and the clotting times in seconds were recorded

Mixture used as a source of antihaemophilic substance	*Substrate used to test the antihaemophilic material*	
	Haemophilic plasma	*Patient s plasma*
Fibrinogen and saline	250	470
Fibrinogen and haemophilic plasma	250	510
Fibrinogen and patient s plasma	1650	1280
No antihaemophilic substance	1620	1330

plasma. In an attempt to locate this inhibitor fractions were prepared from normal and the patient s plasma by Dr R. A Keckwick using the ether precipitation method (Keckwick and Mackay 1949) These fractions were

1 A gamma globulin fraction
2 A beta globulin fraction which also contained some alpha globulin.
3 An albumin fraction which also contained some alpha globulin.
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From tests that were carried out it appeared that the inhibitor responsible for the clotting defect was present in the gamma globulin fraction

When the patient s plasma was mixed with haemophilic plasma there was no shortening of the clotting time on recalcification (Table 37) From this it appears that either the patient's plasma lacks the antihaemophil c globulin or the inhib tor masks its action. The fibrinogen f action isol ted from the patient which was itself only very sl ghtly inhibitory did not shorten the clotting time of haemophilic plasma and therefore did not contain antih emophilic globulin It may be concluded therefore th t the patient's plasma lacked the antihaemophilic

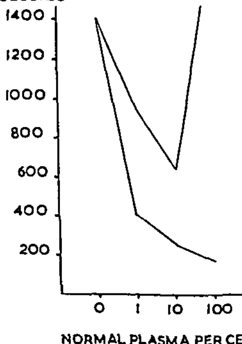


Fig 48 Mixtures of part of normal and part of the patient's gamma globulin fraction were incub ted together for two hours At the end of this time the mixtur was diluted 1/10 and 1/100 with 0 85 per cent saline 0 1 ml. of the dil tions was dded to 0.1 ml. of haemophilic plasma and the mixtures recalcified. The results of this experime t are shown in the upper curve Similar dil tions of untreated normal plasma were tested for comparison (lower curve) It will be seen that th patie t s gamma gl bulin fraction reduced the anti haemophilic effect of normal plasma

globulin and its absence coupled with the presence of an inhibitor naturally suggests a relationship between the two defects If the patient s plasma contained an inhibitor which destroyed or inactivated the antihaemophilic globulin the clotting defect could be explained.

To test this possibility the fibrinogen fraction of normal plasma which contained the antihaemophilic globulin was incubated with the patient s plasma for ten minutes At the end of this time the mixture was added to haemophilic plasma and no shortening of the clotting time of haemophilic plasma was observed (Table 38) From this table it will also be seen that the incubation of the fibrinogen fraction of normal plasma with haemophilic plasma caused no inactivation of the antihaemophilic globulin It appears therefore that a substance is present in the patient s plasma which has the ability to inactivate the antihaemophilic globulin. Further experiments were carried out using the gamma globulin fraction of the patient s plasma which contains a specific inhibitory substance A solution of 50 mg of the patient s gamma globulin fraction in 2 ml. distilled water was incubated at 37 C for two hours with a sample of normal plasma At the end of this time the mixtures were diluted 1/10 and 1/100 and tested for their ability to shorten the clotting time of haemophilic plasma At these dilutions the patient s gamma globulin fraction had no direct inhibitory effect but these dilutions of plasma had practically no ability to shorten the clotting time of haemophilic plasma Presumably the patient s gamma globulin fraction had neutralized the antihaemophilic globulin in the normal plasma (Fig 48)

Precipitin tests were carried out between the patient s plasma and various fractions of normal plasma, but the results were inconclusive

The coagulation defect described in this patient is very similar therefore to that of the patient described by Dreskin and Rosenthal (1950) and the possibility that the previous cases in this group already referred to may have had a similar defect must be considered Unless it is specifically looked for an inhibitor of this type may very easily be missed as it is in itself not immediately very potent in lengthening the clotting time of normal blood It is only after preincubation with normal plasma that its full effect can be seen The relationship to the inhibitor observed in the immunized haemophilic subjects must also be considered In both groups it has been demonstrated in several cases that there is an apparently specific inhibition of the activity of antihaemophilic globulin

MISCELLANEOUS CASES

The third group comprises a number of miscellaneous cases in which transfusion or pregnancy were not involved

One of the first examples of such a condition was described by Joules and Macfarlane (1938) The patient was a woman aged fifty-six who without any apparent cause developed a severe haemorrhagic diathesis with deep tissue haemorrhages involving the joints and the tongue The coagulation time was forty-eight minutes by the Lee and White method and this could be corrected by the addition of 10 per cent of normal plasma The patient s plasma failed to correct the clotting defect of known haemophilic blood and appeared therefore in all respects similar to haemophilic plasma No

positive evidence of a circulating anticoagulant was obtained but incubation with antihaemophilic globulin was not carried out, so that the existence of a relatively slow acting inhibitor of this factor cannot be excluded Pons and Torregrosa (1952) described a very similar case also unassociated with pregnancy in which an anticoagulant apparently active against dilute thromboplastin was demonstrated

Lozner Jolliffe and Taylor (1940) described a sixty-one-year-old mulatto with generalized lymph node tuberculosis and syphilis A haemorrhagic diathesis developed and he died of bleeding following lymph node biopsy The coagulation time was ninety minutes the prothrombin time was normal and there was no evidence of an increased antithrombin The patient s plasma delayed the coagulation time of normal blood and did not shorten the clotting time of haemophilic blood Conley et al (1948) also described a man aged thirty-nine who had syphilis and chronic nephritis and whose clotting time was sixty minutes the patient s plasma lengthened the clotting time of normal blood There was a poor reaction to weak thromboplastin as in haemophilia No precipitins could be detected

Soulier and Burstein (1948) observed a man aged twenty-one in whom the diagnosis had originally been haemophilia There was no family history It was found that his plasma delayed the clotting of normal blood In this case it was observed that the addition of platelets which had been frozen and thawed greatly shortened the clotting time of the patient s blood Antihaemophilic globulin in the form of Cohn s fraction I had no effect and again it was observed that there was a poor clotting response to diluted thromboplastin Quick and Stefanini (1948) and Dieter Spooner and Pohle (1949) described the development of an anticoagulant during the treatment of pemphigus with stovarsol The patient s plasma delayed the coagulation of normal plasma but was not an antithrombin Tzank Soulier and Blatrix (1949) record the existence of a similar anticoagulant in the globulin fraction of the plasma in a case of dermatitis This factor interfered in the reaction of antihaemophilic globulin with the platelets Singer et al (1950) reported a sixty-seven-year-old male in whom the blood coagulation time was 1.0 minutes and whose blood lengthened the clotting time of normal blood The anticoagulant was shown not to be an antithrombin and was apparently an antithromboplastin The clotting defect was

not corrected by adding antihaemophilic globulin but some shortening effect was obtained by adding platelets Harrington et al (1950) describe the case of a woman aged sixty whose plasma lengthened the one-stage prothrombin time of normal plasma This effect was obtained when human thromboplastin was used but not when the test was carried out with thromboplastin derived from animal tissues It appeared, therefore, that this patient s plasma contained a specific inhibitor of human thromboplastin

Abnormal Plasma Proteins

It has also been observed (de Nicola 1950 Bernard et al 1952 André et al 1952 Craddock et al 1953, Gehmacher et al 1954) that in a number of conditions such as liver disease tuberculosis, myelomatosis and neoplasia which are associated with the presence of abnormal plasma proteins (dys-proteinaemias) there is an inhibition of coagulation The main defect seems to be an interference with thrombin generation Mueller Ratnoff and Heinle (1951) describe an anticoagulant observed in the blood of a man suffering from bacterial endocarditis There was an abnormal globulin present in the plasma which precipitated on cooling and thus belonged to the cryoglobulin group The activity of the anticoagulant was more marked at 25 C than at 37° C and it seems likely that the observed effects were due to the abnormal protein

THE MODE OF ACTION OF CIRCULATING ANTICOAGULANTS

Biggs and Douglas (1953b) showed that the presence of a circulating anticoagulant depressed normal thromboplastin formation in the thromboplastin generation test In several cases it was found that the anticoagulant action was apparent even after considerable dilution Hougie and Fearnley (1954) and Hougie (1955a) made a study of the mode of action of anticoagulants in three patients two of whom also had haemophilia They confirmed the finding that the anticoagulant depresses blood thromboplastin formation and showed that if serum antihaemophilic globulin and $CaCl_2$ were incubated together and thereafter the anticoagulant were added no anticoagulant effect could be demonstrated If on the other hand the serum antihaemophilic globulin and $CaCl_2$ were incubated with

the anticoagulant its effect was obvious In other words the anticoagulant inhibited a reaction between antihaemophilic globulin serum and $CaCl_2$ Bergsagel and Hougie (1956) showed that the anticoagulant inhibited the reaction between Christmas factor antihaemophilic globulin and $CaCl_2$ The specific mode of action of the inhibitor supports the view of Bergsagel (1955a and b) that the initial stages of thromboplastin formation involve a reaction between the Christmas factor and antihaemophilic globulin Hougie and Fearnley (1954) could find no difference in the mode of action of the anticoagulants in the blood of haemophilic patients and that of the sporadic case

THE DEMONSTRATION OF CIRCULATING ANTICOAGULANTS

Circulating anticoagulants may be demonstrated by a lengthening of the calcium clotting time of normal plasma on the addition of proportions of the patient s plasma In some cases the inhibitory effect may be obvious only when the mixtures of normal and pathological plasma have been incubated together for some time The anticoagulants can also be demonstrated using the thromboplastin generation test (See Appendix IV 24)

SUMMARY TO CHAPTER XVII

Two main types of anticoagulant may appear spontaneously in the blood of human subjects The first of these is heparin or a heparin-like factor which appears rather rarely in such conditions as anaphylaxis and irradiation and without apparent cause in a small number of other conditions The second type appears to act by inhibiting thromboplastin or the factors concerned in the development of intrinsic thromboplastin In haemophilics immunized by repeated transfusions there is good evidence that the anticoagulant is of the immune body type which combines with or otherwise inactivates the antihaemophilic factor of normal human plasma An anticoagulant has been described in female patients soon after pregnancy and the available evidence suggests that it also is active against antihaemophilic globulin In a third and miscellaneous

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CHAPTER XVIII

THROMBOSIS AND ANTICOAGULANT THERAPY

When normal blood vessels are injured blood clots in them probably because the damaged endothelium forms a foreign surface and causes the activation of intrinsic thromboplastin and the deposition of platelets If the vessels are actually ruptured tissue thromboplastin probably enters the blood The clot does not normally extend beyond the area of damage probably because the coagulant factors concentrated at the site of injury are rapidly diluted in the circulating blood and neutralized In normal people clots do not form in uninjured vessels Pathological thrombosis occurs either when a clot extends beyond a site of injury or when coagulation occurs in uninjured vessels Since the normal fluidity of the blood is determined by a delicately balanced equilibrium it is not surprising that many factors may upset this equilibrium and predispose to thrombosis Severe trauma may predispose to thrombosis by exposing the blood to foreign surfaces causing absorption of tissue thromboplastin and by increasing prothrombin fibrinogen and the number of platelets in the blood Stasis particularly in the legs results from immobilization in bed tight abdominal bandages shallow breathing and cardiac insufficiency This stasis may lead to an abnormally high local accumulation of coagulant factors which if the circulation had been normal would have been diluted and neutralized in the general circulation The increased blood viscosity caused by rouleaux formation may be important in many infective conditions Blood is particularly liable to clot in vessels whose endothelial surface is damaged by disease or whose lumen is diminished by thickening of the wall In a patient in whom enough of these unfavourable circumstances coexist pathological thrombosis may occur

In recent years there has been much interest and speculation about the causes of coronary thrombosis stimulated, presumably by the increasing incidence of the disease and its predilection for professional workers (*Lancet* 1955) There are now many social surveys of the incidence of coronary thrombosis In England its frequency is associated with social class the manual workers being much less

group of cases anticoagulants have been described which appear to be antithromboplastic though not shown to be specifically inhibitors of antihaemophilic globulin The observation by one or two authors that the addition of platelets appears to correct the defect is interesting but at the moment does not appear to give conclusive evidence of the nature of the defect itself Quick's (1951) contention that these cases are due to the presence of an antiplatelet factor does not seem to be in any way proved

rapidly covered by endothelium and incorporated into the vessel wall where they undergo fatty degeneration Illustrations provided by Duguid (1955) suggest that in fatal coronary disease the vessels are gradually occluded by successive episodes of thrombosis and inclusion of the clot into the vessel wall Gofman (1954b) also subscribes to the view that coronary artery disease is a gradual cumulative process ending in coronary occlusion

These various observations provide a very plausible hypothesis about the aetiology of coronary artery disease Too much fat in the diet leads to an increase in coagulant fats in the blood to cumulative atheroma and finally to coronary occlusion However it is very unlikely that the picture is truly quite so simple Morris et al (1953) have shown some correlation between physical activity and coronary thrombosis in workers in similar social groups bus conductors are less likely to get thrombosis than drivers and postmen than other civil servants It is also known that women are less affected by coronary thrombosis than men Atheroma is a generalized disease thus there should also be a parallel increase in cerebral atheroma

Despite these objections the accumulated evidence of a correlation between fat and coronary disease is striking and it is natural to wonder if anything can be done about it Clearly the restriction of fat intake to the level of the Bantu native is undesirable in Britain the fat intake in the war was reduced, 39 per cent of calories being provided by fat in 1938 and 33 per cent in 1947 and the reduction was considered to be a severe hardship Any marked reduction in fat intake for persons over 40 is unlikely to be appreciated Heparin has the ability to reduce the level of β-lipoproteins and Gofman s sf 10-20 lipoproteins in the blood (Kaufmann 1954 Gilbert-Querato et al 1954 Gofman 1954b) However the reduction is very short-lived there is no long term reduction of lipoproteins following heparin therapy (Rinsler 1954) The action of heparin on plasma fat has been studied by French, Robinson and Florey (1953) Robinson and French (1953) and Jefferies (1954) It has been found that heparin activates a plasma lipase lipoid which has become soluble under the influence of heparin can be recovered from the albumin fraction

From the point of view of treatment thrombosis is most important in those patients with cardiovascular disease who have cardiac infarction and in post-operative patients who develop thrombosis The first group of patients are immobilized in bed and usually have some

affected than professional workers (Logan 1952) In Norway during the war there was a fall in the deaths from coronary thrombosis during the German Occupation (Strøm and Jensen 1951, Strøm 1954) In Cape Town it has been shown that coronary thrombosis is most frequent in Europeans very infrequent in the Bantu and of intermediate frequency in the Cape coloured population (Bronte Stewart Keys and Brock 1955 Vogelpoel and Schrire 1955)

These observations naturally suggest some correlation with diet this factor has been studied by Bronte-Stewart et al (1955) in the Cape Town population where it was found that income race and intake of animal fats were correlated the highest consumption of fat being among the European population and the lowest in the Bantu In the war the consumption of fat in Norway was greatly reduced Bronte-Stewart et al (1955) have shown that the serum β-lipoprotein assayed by paper electrophoresis and total cholesteral were correlated with the intake of animal fat being much higher in Europeans than the Bantu and the Cape coloured having intermediate levels Correlation of lipoprotein levels and age have now been carried out Keys et al (1955) have shown that a high cholesterol level is found in people over 40 in populations with a high incidence of coronary thrombosis Gofman (1954b) reports an increasing concentration of a particular group of lipoproteins sf 10-20 (sf = Svedberg unit of flotation, see Gofman 1954a) with advancing years Gofman et al (1951) have also found a high level of sf 10-20 lipoproteins in people with coronary insufficiency when comparison is made with similar people who are not affected High cholesterol values have also been found in these patients by Gertler et al (1950) Steiner (1952) Oliver and Boyd (1953) and Scardigli and Guidi (1954)

Fullerton et al (1953) and O Brien (1955) have shown that high fat meals cause some increased coagulability of the blood indicated by shortening of the clotting time and acceleration of the Russell s viper venom clotting time

To follow the study into the field of morbid anatomy and histology it has been shown by Higginson and Pepler (1953) that severe atheroma a feature of most patients with coronary thrombosis is far less common among the Bantus than in European hospital patients Duguid (1949) (1952) (1955) Ranne and Duguid (1953) have studied the evolution of atherosclerotic lesions both in animals and human subjects Duguid believes with much convincing evidence that atheroma is due to the occurrence of small mural thrombi which are

these patients the evidence for the beneficial effects of anticoagulant therapy is less clear The incidence of thrombosis and its complications differs in different centres Moreover post-operative treatment by early rising active movement the prevention of infection by antibiotic drugs and the prevention of shock by blood transfusion all tend to prevent thrombosis or ameliorate its effects In many series of patients treated with anticoagulant drugs these favourable post-operative conditions have been present and it is impossible to distinguish which of the factors may have been most beneficial

The rationale for anticoagulant therapy is superficially simple Anything which reduces the coagulability of the blood will bias the equilibrium between coagulant and anticoagulant factors in the blood against thrombosis But anticoagulant drugs are often used in doses that have little demonstrable effect on the whole blood clotting time and it is possible that other effects of these drugs are important. Heparin, for example has a vasodilatory effect and dicoumarin may encourage the recanalization of formed clot (Kubik and Wright 1950)

Whatever the value of anticoagulant drugs the reports of their efficiency are enthusiastic and they will certainly be used by clinicians in many types of patient. The dosage of these drugs is controlled by laboratory tests and unless these tests are carried out reliably the effects of treatment may be disastrous The practical use of anticoagulant drugs must therefore be considered in detail.

ANTICOAGULANT DRUGS

The drugs which have been used for anticoagulant therapy can be divided into two groups Those which lengthen the clotting time mainly by influencing the thrombin-fibrinogen reaction (the heparin group of drugs) and those which lengthen the one-stage prothrombin time (the dicoumarin group)

Heparin is a polysulphuric acid ester of a mucopolysaccharide (Chapter VII) Its effect is probably due to a strong acidic charge that it carries Heparin is not effective when given by mouth, and by intravenous injection the lengthening of the clotting time is of short duration 10 000 units prolong the clotting time for 2-3 hours 15 000 units for 4-5 hours and 20 000 units for about 6 hours Thus heparin must be given either by a continuous infusion or by repeated intravenous injections Neither of these methods of administration

degree of cardiac failure These patients are very liable to recurrence or extension of the thrombosis In the second group post operative mortality has been reduced by improved surgical treatment and post-operative care, death from thrombosis and pulmonary embolism though rare has become more important with reduction in other causes of death

It is reasonable to suppose that a reduction of the coagulability of the blood might lessen the incidence of thrombosis or the extension of a thrombus once formed There is now no doubt that anticoagulant therapy reduces the mortality of patients with coronary thrombosis When the figures for treated and untreated series of patients are compared (Table 39) it will be seen that the number of deaths are reduced to about half by anticoagulant therapy

In post-operative patients there are many enthusiastic claims that anticoagulant therapy has reduced the morbidity and mortality In

TABLE 39

PATIENTS TREATED WITH ANTICOAGULANT THERAPY

	Number of Ca es		*Mortality (total)*	
	Control	*Treated*	*Control*	*Treated*
Wright et al (1948 and 1950)	368	432	88	65
Tulloch and Gilchrist (1950)	84	70	34	16
Gluck et al (1948)	25	25	8	3
Greisman and Marcus (1948)	100	75	35	7
Hilton et al (1949)	19	38	9	5
Peters et al (1948)	*86*	*110*	22	*12*
Parker and Barker (1947)	100	50	13	5
Freston and Taylor (1950)	54	45	1	9
Zeluff and Field (1950)	100	80	40	20
Totals	93(	925	27 7 /	15 35 /

(Wright 1954) reduce the effectiveness of platelet thrombi (Zucker 1947) increase the speed of recanalization of thrombosed vessels (Wright 1954) and cause some increase in capillary fragility particularly in toxic doses The antithrombosis effects of the dicoumarin drugs may be at least in part due to changes other than those of the clotting mechanism Similarly tendency to bleed may be caused as much by the capillary abnormality as by the alteration in clotting

For many years dicoumarin was the only drug of this type in general use but recently very many synthetic compounds have appeared the chemical relationships of these different drugs has been reviewed by Hunter and Shepherd (1955) so far no correlation between chemical structure and anticoagulant action has been found Clinically the drugs differ from each other in the size of the dose required and in the duration of effectiveness

Dicoumarol (3-3′ methylene bis 4 hydroxycoumarin) the full chemical formula of which is given on page 213 is still in use together with many synthetic derivatives including

Tromexan in which the methylene carbon of dicoumarol carries the ethyl ester of a carboxylic acid group

Marcoumar in which the methylene group carries a substituent ethyl group and the second coumarinyl group is replaced by an unsubstituted phenyl

Warfarin in which the substituent ethyl group of Marcoumar is replaced by acetonyl

Geigy compound 23350 in which the phenyl of Warfarin is replaced by p-NO_2 phenyl

In *Cumopyran* the hydroxyl group of the 4-hydroxycoumarin and the methylene carbon of the compounds described above become part of a second heterocyclic ring Its formula is

3 4-(2′-methyl-2 methoxy-4′phenyl) dihydropyrano-coumarin

Dindevan (2-phenylindane 1 3 dione) has lost the heterocyclic oxygen characteristic of coumarin although its formula still bears a close resemblance to the series in having its substituent oxygens and aromatic ring in the same relative positions

METABOLISM AND EXCRETION OF ANTICOAGULANT DRUGS

Heparin which is injected intravenously in high concentration leaves the circulation at the rate of about 3-5 units/kg/minute but the rate of elimination falls progressively with lower concentrations Relatively small doses of heparin given to experimental animals are

is convenient and this difficulty of administration and the expense of the drug are serious disadvantages to its use A concentrated heparin solution which can be given by intramuscular injection has also been used and this gives satisfactory results but the injections are painful Attempts have been made to combine heparin with a medium which delays absorption from intramuscular injections (Evans and Bollar 1946 Goodwin and Macgregor 1950) but these injections are also often painful It has been suggested that heparin is absorbed from the mouth if small wafers containing heparin are allowed to dissolve under the tongue (Litwins et al 1951) but this claim has not been confirmed

Attempts to synthesize less expensive drugs allied to heparin have not in the past been very successful Piper (1945 1946) Astrup and Piper (1946) made cellulose and chytin polysulphuric acid esters but these were very toxic when injected into animals they caused collapse and a removal of the platelets from the blood and precipitation of fibrinogen A polysulphuric acid ester of polyanhydromannuronic acid called paritol has been used in human patients but there was a high incidence of toxic reactions (Sorenson and Wright 1950) Of thirty-five cases treated one patient had severe and immediate collapse two developed swelling of hands and feet and two patients had painful extravasations round the site of administration Synthetic compounds including paritol and thrombocid tested by Astrup (1953) could all be shown to cause in vitro clumping of platelets Experiments with dextran sulphates have been more hopeful (Walton 1951) It was found that compounds with a molecular weight of over 20 000 were toxic and produced precipitation of fibrinogen and platelet agglutination Compounds with a molecular weight of less than 20,000 had no toxic effects in animals and lengthened the clotting time for periods of time up to two to three hours A clinical trial of these compounds is being undertaken and when the results are available these low molecular weight dextran compounds may be found to be suitable to replace heparin

The second group of anticoagulant drugs, the dicoumarin group has a complex effect on the clotting mechanism There is an early fall in Factor VII and some factor or factors affecting thromboplastin formation and a later and usually less important fall in prothrombin level as measured by the two-stage area method marked reduction in prothrombin usually occurs only with gross overdosage In addition the dicoumarin drugs reduce the adhesiveness of platelets

From this work a fairly clear sequence of cause and effect emerges The different drugs vary in their speed of absorbtion into and elimination from the bloodstream following this the prothrombin time response varies both in its speed of onset and duration Superimposed on this predictable general response there is a very wide unexplained individual variability in the degree of response both in different patients and in the same patient at different times

Another important feature of this study is the observation that the metabolism of the drugs in various species is quite different Thus for example, one compound may disappear from rabbit blood at the rate of 50 per cent per hour and from dog blood at 4 per cent a day The breakdown products appearing in the urine are also quite different in different animals Thus no conclusions about the probable behaviour of these drugs in human subjects can be deduced from animal experiments

CHOICE OF ANTICOAGULANT DRUGS

There are several commercial preparations with a heparin-like action and innumerable drugs of the dicoumarin type The choice of heparin-like drugs is fairly simple no drug should be used clinically which has been shown to agglutinate platelets in vitro (paritol thromboaid) There remains two possible drugs heparin and dextran sulphate Of these heparin is still probably the best having been used over a much longer period of time Usually these drugs are used to cover the initial period before the dicoumarin drugs become effective Of the dicoumarin drugs the choice is more difficult and largely a matter of individual preference In practice the choice rests between short acting drugs which are rapidly absorbed and eliminated and long acting drugs which vary in their speed of absorption but which are slowly eliminated The rapidly eliminated drugs have the disadvantage that frequent doses are required and the fluctuations in blood level may make it difficult to maintain a steady prothrombin time response They have the advantage that cessation of the drug is quickly followed by a return to normal clotting function Sometimes a rapidly acting drug is used for the initial period of treatment only though this method is likely to delay stabilization The long acting drugs which are also slowly absorbed have the disadvantages that clinical effect is not achieved for several days (unless the first few doses are given intravenously) there is serious danger of overdose in sensitive patients during this phase and of cumulation during

eliminated normally after nephrectomy and evisceration suggesting that neither the kidneys nor the gastro-intestinal tract are essential for the removal of heparin from the blood If larger doses are used (150 u/kg) evisceration and hepatectomy interfere markedly with the removal of heparin If heparin is given intramuscularly nephrectomy may greatly reduce its elimination Examination of the urine after heparin injections shows that no heparin appears in the urine unless doses of more than 400 u/kg are given, higher doses lead to increasing amounts in the urine Examination of tissues particularly liver, has shown that these contain an enzyme heparinase which can destroy heparin This evidence given by Jaques Bell and Cho (1954) suggests that heparin is normally largely destroyed by an enzyme in the tissues and that elimination in the urine only becomes important when very large doses of heparin are used These experiments are necessarily done with single doses of heparin whereas human subjects receive repeated doses It would be very unwise to assume that the human subject would eliminate heparin normally with severe kidney failure It is our experience that heparin may accumulate dangerously in such patients From the experiments of Jaques et al (1954) it would obviously be unwise also to give large doses to patients with severe liver failure because heparinase activity might be much reduced

The metabolism of the dicoumarin drugs has recently been studied and a report of this work is given by Weiner Brodie and Burns (1954) These authors have studied the blood levels of the dicoumarin drugs and the products which appear in the urine They have found that in general the time during which the drug remains in the blood is related to its duration of action Thus the drugs of long action remain long in the blood stream Individual responses complicate the study but from carefully controlled experiments the relative speeds of disappearance can be studied For example tromexan a very rapidly eliminated drug leaves the circulation at the rate of 25 per cent per hour, whereas warfarin a drug of protracted action disappears from the blood at the rate of 17 per cent per day The prothrombin time response follows one to two days after the peak level of drug in the plasma The speed of absorbtion of the drug into the plasma is also variable and this naturally controls the speed of response During the investigation it was found incidentally that some tablets were so firmly compressed that they failed to dissolve during digestion and very little drug was absorbed

daily may be necessary on the other hand with warfarin or dipaxin doses every three to four days or weekly may achieve the same result With long acting drugs it may be safer to use this intermittent type of dosage rather than small daily doses if the prothrombin time is allowed to decrease slightly before each new dose the danger of cumulation is less This regime requires frequent prothrombin time estimations A feature that has recently been emphasized is that the dose required by a patient may be very critical A difference in effect between 75 and 100 mg of dindevan daily may be marked The only easy way to adjust the dose is to keep a graphical record of dose and response and gradually to reduce the fluctuations in prothrombin time by using smaller dose differences

It is of course widely appreciated that patients vary in their sensitivity to these drugs and one patient may vary as greatly from one time to another

CONTRAINDICATIONS TO THE USE OF ANTICOAGULANT DRUGS

Since the choice of patients for anticoagulant therapy is a clinical decision which must depend on the individual physician s belief in its efficiency it is not within the scope of this book to discuss cases in which the drug *should* be used From the mode of action and excretion of the drugs it is possible to give a number of general rules about patients to whom the drugs may be unusually dangerous

The most important group are patients who have some other predisposition to haemorrhage Among these are patients who have or have recently suffered from peptic ulceration patients with carcinoma or polypi in the gastro-intestinal tract patients who have had a cerebrovascular accident patients with malignant hypertension or retinal haemorrhages and patients with polyarteritis nodosa

Conditions which might interfere with the metabolism or excretion of the drug are known liver disease or renal failure from any cause Patients with congestive cardiac failure must often be treated with anticoagulants though both hepatic and renal function may be impaired These patients may prove very sensitive to small doses of drug but provided this is recognized there is no excessive danger

DURATION OF THERAPY

Patients who have had coronary thrombosis are usually treated with anticoagulant drugs in hospital for 3-8 weeks The value of this

maintenance therapy On the other hand, skilful use of these long acting drugs for maintenance may result in very constant prothrombin time levels The dangers of overdosage and cumulation are reduced by the availability of Vitamin K_1 which usually acts rapidly in shortening an excessively prolonged one-stage prothrombin time This treatment does not entirely remove the risk of overdosage because the level of prothrombin as judged by the two-stage method may not respond very rapidly to Vitamin K_1

There is no single preferable course but it should be noted that any mode of treatment is greatly benefited by the physician s experience in handling a particular drug, unsatisfactory results are likely to follow changes from one drug to another as new compounds become available In this country dindevan (phenylindanedione) is probably the most favoured drug it is of intermediately rapid action and duration of effect

THE DOSAGE OF ANTICOAGULANT DRUGS

Heparin is usually given by intravenous injection in doses of 10 000-15 000 units 4-6 hourly

No general rules about dosage and regime to be followed using the dicoumarin drugs can be given Doses which give approximately the same final prothrombin time response together with a record of speed of action and rate of recovery are given in Table 40 To maintain steady dosage with tromexan, doses three to four times

TABLE 40

DURATION OF PROTHROMBIN RESPONSE AFTER SINGLE DOSES OF VARIOUS AGENTS IN MAN (DOSES COMPARED ARE THOSE WHICH GIVE THE SAME ULTIMATE PROTHROMBIN TIME RESPONSE CONTROL 13 5 SECONDS PATIENT 23 SECONDS) FROM WEINER BRODIE AND BURNS (1954)

Drug	*Dose mg*	*Day of Prothrombin time*	
		Peak	*Recovery*
Tromexan	1650	1	2
G 23 350	20	1 2	3
Phenylinaned one	350	2	4
Dicoumarol	400	2-3	5
Cumopyran	150	2 3	5-6
Warfarin	65	2 3	5-6
Marcoumar	24	2-3	6
Dipaxin	20	3 4	7

TABLE 41

LONG TERM ANTICOAGULANT THERAPY

Author	Condition	Control Observations			Treatment Observations			
		Number of Patients	Duration of Observation (months)	Emboli Thrombosis	Number of Patients	Duration of Treatment (months)	Emboli Thrombosis	Deaths
Foley et al 1954	Rheumatic Heart Disease	29	768	117	29	1128	18	6
Owren 1955	Rheumatic Heart Disease	—	—	—	13	295	1	
Foley et al. 1954	Recurrent Thrombophlebitis	24	2207	92	24	896	7	1
Foley et al. 1954	Myocardial Infarction (2 episodes)	11	587	49	11	393	3 }	8
	1 episode	12	41	12	12	554	1 }	
Owren 1955	Angina No previous Infarct	—	—	—	128	3888	10	12
	Infarct	—	—	—	108	3420	7	12

short-term treatment is now seldom questioned It has always been realized that many of these patients return to hospital later with a second coronary thrombosis and required another course of anti coagulant therapy In some centres the opinion is growing that these and other patients with a special liability to recurrent thrombosis or embolism should be treated permanently on anticoagulant drugs The technical and administrative difficulties of such long-term treatment are great and the evidence about its beneficial effects must be examined carefully Difficulty in assessing such evidence arises from the apparently variable prognosis from one centre to another and from ethical doubts about maintaining an adequate control series

Follow-up studies on patients with coronary thrombosis who have received one course of anticoagulant therapy suggest that 15-18 per cent of the patients die in the first year but having survived one year the prognosis is better 8-10 per cent of the surviving patients dying in the second year (Katz et al 1949 Block et al 1952 Owren 1955) Thus although survival of a first attack is greatly improved by short term anticoagulant therapy the ultimate prognosis remains poor These findings naturally suggest that long-term therapy for patients with a predisposition to episodes of thrombosis might be valuable Owren 1954b and c 1955) has treated more than 400 patients on long term therapy and the death rate is 4-5 per cent per year where very roughly comparable control series show 8-10 per cent The two series are not really comparable because they were not randomly selected It is Owren's opinion that there is a reduction to half in the death rate per year in patients treated on anticoagulants as compared to untreated patients In another major study Foley et al (1954) have attempted to control their results in treated patients by the patient's record while under observation but not treated (Table 41) This method may be reasonable for the patients with rheumatic heart disease or recurrent thrombophlebitis but it is to be doubted if it is in any way valid for patients who have had one coronary thrombosis These patients are selected precisely because they have had one thrombosis the time which might elapse even without treatment before a second thrombosis occurred is quite unpredictable This study does certainly suggest a great reduction in thrombo-embolic episodes in patients with rheumatic heart disease and recurrent thrombophlebitis The experience of Burt (1954a and b) suggests that long-term therapy is beneficial in recurrent thrombophlebitis

The general probability suggests that long-term anticoagulant

effect on the one-stage prothrombin time but all of which may predispose to bleeding The use of the one-stage prothrombin time relies on a reasonable correlation between Factor VII reduction and the other effects of the drugs It is clear that this correlation is not very close It is for this reason that there have been recent attempts to replace the one-stage test by other techniques This problem will be discussed later at the present there is no test which can replace the one-stage prothrombin time for routine use and the important practical issue is to determine the most reliable form of the one-stage test

THE UNMODIFIED ONE-STAGE TEST

Since this test is very widely used it is natural that many modifications should have been made It is important to appreciate the technical factors which affect this test because these are more or less important in the original test and in various modifications and will affect the reliability of the method The technique of Quick s original test has been outlined in Chapter XI and is given in detail in Appendix IV 7 The main factors which affect the unmodified technique will now be discussed

THE EFFECT OF CALCIUM CONCENTRATION

The concentration of calcium must be optimum The optimum range for normal plasma is wide but it is much narrower for pathological plasma To determine the practical optimum it is necessary to use pathological plasma In our experience an M/40 concentration of $CaCl_2$ is adequate

THE EFFECT OF TYPE AND CONCENTRATION OF THROMBOPLASTIN

The type of thromboplastin used makes an enormous difference to the test Russell s viper venom for instance is insensitive to Factor VII concentration and reflects mainly the prothrombin level in the plasma and it should never be used to control the dicoumarin drugs Thromboplastin from different sources and even different batches of brain extract from the same tissue and species may lead to considerable differences in interpretation (Merskey and Scholtz 1955 Hecht 1955) These variations cannot easily be eliminated in the unmodified test and are a constant source of anxiety to those responsible for routine testing

The concentration of a particular thromboplastin may also be

therapy might be effective, the observations which exist support the hypothesis proof, for patients with coronary thrombosis must await the study of an adequately controlled series

LABORATORY CONTROL AND TREATMENT

The purpose of laboratory control is to ensure that the dosage of drugs for a particular patient is neither inadequate nor excessive Heparin therapy is controlled by the whole blood clotting time if the objective of treatment is to lengthen the clotting time over the whole 24-hour period dosage may be controlled by carrying out the clotting time test just before a dose In practice the dose of heparin used seldom does lengthen the clotting time for the whole 24-hour period and yet the clinical impression is that the treatment is satisfactory In most cases therefore no laboratory control is required

With the dicoumarin drugs the usual policy is to give the maximum dose compatible with safety The main purpose of the labor atory testing is therefore to distinguish those patients in whom haemorrhage may occur The most commonly used test is the one-stage prothrombin time test of Quick (1935) With this test it is possible to divide patients into two groups, one in which haemorrhage is very unlikely and a second in which haemorrhage is rather more probable Thus if the one-stage prothrombin time of the patient's plasma is less than 3 times as long as that of a normal plasma haemorrhage is unlikely If the prothrombin time is more than 3 times normal haemorrhage may occur This rather crude separation is not very satisfactory There is no means to telling which patients whose prothrombin time is excessively long will bleed Some patients are accidentally overdosed for weeks with no *bleeding* some patients bleed immediately their prothrombin time is long some patients will have no bleeding for 3 or 4 days of lengthened prothrombin time and then suddenly bleeding will start A physician *may observe many instances in which a lengthened* prothrombin time has been harmless and an unwarranted sense of security may be encouraged and little attention paid to occasional overdose

This rather poor correlation between one-stage prothrombin time and bleeding is probably due to the multiple effects of the dicoumarin drugs The one-stage test mainly measures Factor VII activity but the dicoumarin drugs affect in addition capillary fragility platelet adhesiveness and thromboplastin formation none of which have any

include a comparison with normal This comparison has been made in one of three ways

A dilution curve of normal plasma is constructed and the results of the abnormal specimen read from the curve By this method the clotting time shows little change until the dilution has reached 20 per cent thereafter reduction in the proportion of normal plasma greatly affects the clotting time (Table 42) In practice it is found

TABLE 42

COMPARISON OF DIFFERENT METHODS OF RECORDING THE RESULTS OF THE ONE-STAGE PROTHROMBIN TIME

Dilution curve Method			*Prothrombin Index using Clotting Time Figures of*		*Ratio of Clotting Times using the Clotting Time Figures of*	
Percentage	*Equivalent Clotting time (Quick 1942)*	*Equivalent Clotting time (Biggs and Macfarlane 1949)*	Quick (1942)	*Biggs and Macfarlane* (1949)	Quick (1942)	*Biggs and Macfarlane* (1949)
100	12	14	100	100	1 0	1.0
50	16	16	75	88	1 3	1 1
20	26	21	46	66	2 2	1 5
10	38	29	31	48	3 2	2 0
5	70	46	17	30	5 8	3 3

that the shape of the dilution curve varies very much from one centre to another and even with different batches of thromboplastin in the same centre Thus 'percentages determined in this way give little clue to the actual lengthening of the clotting time though with much patience and ingenuity the results may be consistent in any one laboratory

A second method is to express the results as an index The clotting time of the normal plasma is divided by that of the abnormal and the result given as a percentage Thus if the normal plasma clots in 12 seconds and that of the patient in 24 seconds the prothrombin index would be said to be $\frac{12}{24} \times 100 = 50$ per cent This method of calculating a percentage' obviously bears no relation to the dilution curve method yet results are often expressed as per cent no indication of the method of arriving at the figure being given

The confusion about percentages has lead many workers to use a

important Some preparations are inhibitory in high concentration and the optimum concentration for a particular batch should be determined

THE EFFECT OF CONCENTRATION OF VARIOUS FACTORS IN THE PLASMA

The one-stage test is greatly affected by the levels of Factors V and VII Factor V is not influenced by the dicoumarin drugs The clotting time is also affected by the level of fibrinogen if this falls below 100 mg/ml This factor is seldom confusing in the unmodified test

THE EFFECT OF STORAGE OF SPECIMENS

Citrated plasma samples change on storage the one-stage prothrombin time becoming progressively shorter probably due to activation of Factor VII Oxalated samples have a lengthening of the one-stage time on storage due to deterioration of Factor V It seems unlikely that the test would be useful for postal samples

THE EFFECT OF HEPARIN

Heparin affects the one-stage prothrombin time and it is not possible to obtain reliable values for the one-stage prothrombin time in the presence of heparin

THE APPLICATION OF THE ONE-STAGE TEST IN THE CONTROL OF THERAPY

This test has been used so extensively in the control of therapy that its value can scarcely be doubted It has the advantage of technical simplicity but cannot be used if heparin is also present in the blood and cannot be carried out on postal samples because Factor V may deteriorate in oxalated blood and the one-stage prothrombin time of citrated plasma may shorten on storage Thus patients on long-term therapy presumably cannot have holidays Differences in the effects of various thromboplastin preparations mean that results from different centres may not be exactly comparable Indeed Smith and Schelling (1954) have shown that the criteria adopted by different centres are not the same

The first difficulty in interpretation arises from the fact that the clotting time of normal plasma depending on the activity of the thromboplastin used varies from 12 to about 20 seconds The results of the one-stage test cannot be expressed as clotting times A clotting time of 24 seconds would be quite abnormal for a system with an active thromboplastin (12 seconds normal) but nearly normal for an inactive preparation The results must therefore

The method as proposed by Owren and Aas is known as the p and p method because it is thought to give a combined estimation of the levels of prothrombin and proconvertin (Factor VII) In this method a 1 in 10 dilution of plasma is mixed with prothrombin-free ox plasma (which contains Factor V and fibrinogen) brain extract and

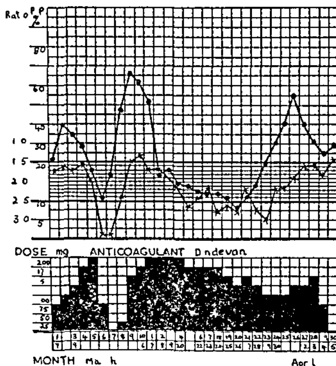


Fig 49 A comparison of the unmodif d one-st ge proth ombin time and the p and p method of Owren and Aas (1951) for the control of anticoagulant ther py The results of the one-stage test are expressed as ratios and those of the p and p m thod s per cent The dosage of dindev n is given above The proposed therapeutic ranges f r the two methods are indicated by the cross-matched areas

In general it is cle r that the two methods e measuring the same bnormality the same trends are obvious in the two methods and high ratios coincide with low percentage figures During the first 6 days of treatment the dindevan is cumulating a fact which is obvious in the p and p method but only revealed in an exaggerated form on the 6th day by the one stage method Therapy was controlled by the one-stage method and the excessive ratio lead to *withdrawal of the drug an entirely unnecessary* precaution as judged by the p and p method. After the seventh day th dose was gradually increased to a value only slightly too high. Too great a dose reduction f llowed between the 19th and 26th day which was immediately obvious using the p and p m thod but not by the one-stage method The patient s balancing

direct ratio of abnormal and normal clotting times to avoid this confusion By this method an abnormal result of 24 seconds would be compared with a normal of 12 seconds by dividing one by the other to give a ratio of 2 Table 42 gives a rough comparison of the results as judged by the three methods

Of these three methods the ratio method is probably the least ambiguous and easiest to use In practise it is usual in establishing the method for control of therapy to adopt a fairly arbitrary standard for the therapeutic range and the level which should not be exceeded based on published figures The method is then used for a period of time and if several episodes of bleeding are observed at apparently safe levels the criteria are readjusted (Merskey and Scholtz 1955) In our laboratory we recommend a therapeutic range of ratios between 1 5 and 2 5 and set the danger level at a ratio of 3

MODIFICATIONS OF THE ONE-STAGE PROTHROMBIN TIME

MICROMETHODS

As usually performed the one-stage prothrombin time involves the use of venous blood and many workers have felt that it would be an advantage to use capillary blood Unfortunately, none of these methods is sufficiently reliable

DILUTION METHODS

The unmodified one-stage test gives little changes in clotting time for reductions in Factor VII down to 20-25 per cent It is often thought that this insensitivity is unimportant because the test is reasonably sensitive within the therapeutic and toxic ranges But many workers have felt that the test would be more useful if minor deviations from normal were more accurately determined because trends towards or out of the therapeutic range would be more obvious Simple dilution methods have the disadvantage that Factor V and fibrinogen are diluted and physiological variations in these factors have an unpredictable effect causing a considerable increase in random and irrelevant variation Owren and Aas (1951) have introduced a dilution method in which these errors are eliminated by making a constant addition of prothrombin-free plasma in which Factor V and fibrinogen are present in high concentration

TESTS OTHER THAN THE ONE-STAGE PROTHROMBIN TIME

The rather poor correlation between tendency to bleed and the results of the one-stage prothrombin time has lead to the search for other tests which might be used to control therapy The heparin resistance test now has many advocates The test is simple to do but is greatly influenced by very many irrelevant factors and is at present impossible to standardize for routine use Theoretically the test has the advantage of being influenced by all stages of clotting it should therefore record the thromboplastin defect caused by the dicoumarol drugs which is not measured by the one-stage test The ingenious Thrombelastograph of Hartert (1951 1952a b and c) also has possibilities for the control of anticoagulant therapy The method has not yet had an adequate trial in parallel with other methods and requires an expensive apparatus

TABLE 43

HAEMORRHAGIC COMPLICATIONS OF ANTICOAGULANT THERAPY

Author	*Type of Treatment*	*Number of Cases*	*Number of Treatment Years*	*Number of Haemorrhagic Incidents*
Wright et al (1948)	Short Term	368	—	30
Beaumont et al. (1952)	Short Term	450	—	36
Owren (1955)	Short Term	1053	110	0
Nichol and Borg (1950)	Long Term	78	—	13
Foley and Wright (1954)	Long Term	85	300	31
Owren (1955)	Long Term (Dicoumarol)	236	607	17
	Long Term (Dindevan)	183	109	1

dose of dindevan is clearly about 150 mg daily The patient's dosage was never truly controlled in spite of 31 days of experience

Had the p and p method been used the dose on the 5th day would have been adjusted to 100–150 mg instead of being increased to 200 mg and the balancing dose would have been reached within the first week

This case was selected because it illustrates many points but the record is in no way exceptional Firstly it is clear that whatever control method is used correlation of dose and response cannot be achieved without a graphical record Secondly balanced dosage requires a reliable record of trends which are not obvious in the one stage method Thirdly the deficiency of the one-stage method is due mainly to its extreme insensitivity to near normal values

The form used for the preparation of this figure is one which could well be used for routine control of therapy The scale markings are left blank on the printed sheet to allow for the use of different drugs and different laboratory methods

$CaCl_2$, the clotting time is recorded as with the one-stage prothrombin time and the results assessed from a dilution curve of normal plasma The method has the initial advantages that postal samples can be tested because deterioration in Factor V is unimportant Factor V being added in excess Heparin in low concentration does not affect the result because a 1 in 10 dilution of plasma is used Also it is claimed that different batches of thromboplastin do not affect interpretation There is also no doubt that the method is very much more sensitive to minor deviations from normal than is Quick's original method Experience with this method used in parallel with the unmodified test suggest that trends in the level of abnormality are earlier and more reliably indicated by this dilution method that this method has less random variation and that its results are earlier and more closely correlated with dosage alterations These points are illustrated in Fig 49 a detailed comment on which is included in the legend

With all these advantages should this method be generally adopted? For hospital in-patient treatment the unmodified test though undoubtedly more difficult for the clinician to use than the method of Owren and Aas (1951) is probably adequate For long-term out-patient treatment there can be no reasonable doubt that the method of Owren and Aas is preferable This belief is supported by the fact that Owren has a very low incidence of haemorrhagic complications in the largest series of cases treated over extended periods yet available His figures show an incidence of haemorrhage of 1 3 episodes per 100 patient-treatment-years where a comparable figure for the American series of Wright was 125 The American series dealt only with in-patients whose prothrombin time levels are more easily controlled

used as thromboplastin and this modification of the test is quite unreliable for the control of anticoagulant therapy Again the test may be adequately performed but the results presented to the doctor in charge of the patient in a form which may be misinterpreted The result may appear as say, 10 per cent The doctor in charge of the patient may have the impression that the therapeutic range is 10-20 per cent and consider the result satisfactory But the therapeutic range of 10-20 per cent refers to a particular dilution curve used by a particular laboratory and if the 10 per cent result is an index figure (10 times the normal clotting time) it will indicate gross overdosage

INADEQUATE SUPERVISION OF THE PATIENT

Hospital in-patients suitably selected seldom suffer from seriously inadequate supervision though there is no doubt that treatment in most hospitals could be much improved by the early appreciation of a trend towards overdosage which is very evident when the p and p method is in use This trend is greatly clarified by the use of graphical representation of results and dosage and this method should always be used

In out-patients the problem is much more difficult and there are many causes of accidental overdose Now that many different drugs are available a different drug of smaller dose may be substituted with the wrong dosage level on renewing the pills (Hunter and Walker 1954c) The patient may be told to stop taking the pills but attributing his improvement to the medicine may continue to take them secretly if he has a large supply in reserve The patient s sensitivity to the drug may suddenly increase either spontaneously or in association with some intercurrent illness or because he has taken some other drug such as aspirin or sulphonamide which potentiate the action of the dicoumarin drugs The patient may have had a healed gastric ulcer unsuspected during in-patient treatment which may recur and bleed during prolonged out-patient therapy The patient may suddenly be taken with tooth-ache and undergo dental extraction without the knowledge of the supervising physician The physician may have seen many patients who have come to no harm through moderate overdose and he may fail to take an appropriately serious view of an occasional prolonged one-stage prothrombin time

These dangers can be minimized by good co-operation between the laboratory and physician and by careful but time consuming

THE HAEMORRHAGIC COMPLICATIONS OF ANTI COAGULANT THERAPY

It is often thought that heparin occurring naturally and being rapidly eliminated is a safe anticoagulant In practise heparin is just as likely to cause bleeding as the dicoumarin drugs and just as much care must be used in selecting patients for treatment

The incidence of bleeding episodes in different series using the dicoumarin drugs is very variable (Table 43) In Owren s series of more than 1000 cases of short-term treatment no haemorrhage attributable to the anticoagulant therapy was observed In Wright s series 36 incidents were observed in treating 450 patients The incidence of bleeding is probably determined by the skill of medical supervision correct selection of patients and the adequacy of the method of laboratory control Owren attributes his low incidence of haemorrhage largely to the method of laboratory control

The most usual type of bleeding is haematuria epistaxis, bruising purpura and gastro-intestinal bleeding are also encountered

THE CAUSES OF INADEQUATE CONTROL AND OVER DOSAGE WITH THE DICOUMARIN DRUGS AND METHODS OF AVOIDING THEM

Many circumstances make anticoagulant therapy *difficult* and dangerous This treatment is superficially very simple but requires supervision by specially trained workers The main causes of poor control are inadequate laboratory methods and inadequate supervision

INADEQUATE LABORATORY METHODS

The best available method for the control of anticoagulant therapy is the one-stage prothrombin time but some workers have attempted to use such methods as the Lee and White clotting time or calcium clotting time Both of these tests give entirely normal results with the blood of patients treated with the dicoumarin drugs unless a gross overdose is given The use of these methods is therefore almost always attended by uncontrollable bleeding

If the correct method is used its performance or interpretation may be unsatisfactory For example Russell s viper venom may be

that may be required Gastro-intestinal bleeding with no other haemorrhage at a therapeutic level always suggests some complicating lesion such as a gastric ulcer The drug should be stopped and Vitamin K_1 given as anticoagulant therapy is contra-indicated in such patients Generalized or severe local bleeding should be treated by withdrawal of the drug with Vitamin K_1 and if necessary by blood transfusion Water soluble Vitamin K preparations are ineffective Blood transfusion is indicated for excessive blood loss blood by itself does not greatly influence the clotting defect. If accidental severe overdose has been given the two-stage prothrombin test should be done during the recovery period the prothrombin level may return to normal more slowly than is suggested by the reduction in one-stage prothrombin time

SUMMARY TO CHAPTER XVIII

Much attention has recently been given to the increased incidence of coronary thrombosis There is some evidence that it may be associated with some abnormality in fat metabolism

Two types of anticoagulants are used for the treatment of thrombosis The heparin group has a limited application and at present heparin itself is the only generally useful drug The dicoumarin group now includes many drugs and the choice between them is largely a question of personal preference

Work on the metabolism of the dicoumarin anticoagulants suggests that the different drugs are not metabolized in the same way and that different animals metabolize the drugs differently

There is now little doubt that short-term treatment with the dicoumarin drugs is of benefit to patients with coronary thrombosis Many physicians believe that long-term treatment would be even more valuable

For the laboratory control of anticoagulant therapy no method is superior to the one-stage prothrombin time It seems probable that the modification of this test described by Owren and Aas (1951) is superior to all other tests available at present.

supervision The laboratory should leave the physician in no doubt as to the interpretation of results The first result obtained for a new patient should be accompanied by a note on its interpretation For example, if the ratio method is used the note may read The results of the one-stage prothrombin time are expressed as a ratio of the clotting times of abnormal and normal plasma samples By this method the therapeutic range lies between 1 5 and 2 5 Values above 3 may be associated with haemorrhage this value should not be exceeded The interpretation of results by any method is much facilitated by graphical representation and routine sheets suitable for this purpose should be available A possible form adaptable to different testing methods was used in the preparation of Fig 49 The control of dosage should be in the hands of an experienced senior member of the staff and not decided by relatively inexperienced workers There is much to be said for the control of dosage in a particular hospital being in the hands of one specially experienced physician He need not be responsible for the patient s care but merely act in an advisory capacity suggesting the best dose each day Out-patients must be very carefully upervised and failure to attend at the laboratory or for an out-patient appointment should be investigated immediately Patients must understand and be capable of understanding the treatment that they are receiving Holidays require careful planning either the patient must attend at a hospital while away or a method must be selected which can be used on postal samples and his dose must be arranged by telephone or post. A patient must be able to come up to hospital at any time to report any sign of haemorrhage and his records must be readily available so that the cause of bleeding is obvious and suitable treatment may be given even if the physician in charge is not to be found

THE TREATMENT OF HAEMORRHAGE

A one-stage prothrombin time should be carried out immediately on the blood of any patient receiving anticoagulant therapy who is bleeding or has petechial haemorrhages or ecchymoses The results of the test and the severity of the bleeding will determine treatment If the bleeding is confined to slight haematuria and the one-stage prothrombin time exceeds the therapeutic range withdrawal of the drug and its cautious re-administration at a lower dosage is all

that may be required Gastro-intestinal bleeding with no other haemorrhage at a therapeutic level always suggests some complicating lesion such as a gastric ulcer The drug should be stopped and Vitamin K_1 given as anticoagulant therapy is contra-indicated in such patients Generalized or severe local bleeding should be treated by withdrawal of the drug with Vitamin K_1 and if necessary by blood transfusion Water soluble Vitamin K preparations are ineffective Blood transfusion is indicated for excessive blood loss blood by itself does not greatly influence the clotting defect. If accidental severe overdose has been given the two-stage prothrombin test should be done during the recovery period the prothrombin level may return to normal more slowly than is suggested by the reduction in one-stage prothrombin time

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CHAPTER XIX

ARTIFICIAL COAGULANTS AND HAEMOSTATICS

The clotting of normal blood can be hastened by adding to it natural or artificial coagulants or by providing a large area of contact with a foreign surface Such procedures are often used in an effort to control persistent or profuse haemorrhage and since the mechanism of haemostasis is a complex one capable of being influenced in many different ways an almost endless series of haemostatic agents have been devised and recommended by their sometimes over-optimistic originators In this chapter will be considered the probable mode of action of agents known to accelerate blood coagulation in vitro and some of the procedures which may increase the coagulability of the blood in vivo A second section will deal briefly with haemostatics that have been used in practice

COAGULANT PREPARATIONS

SUBSTANCES HAVING A THROMBIN-LIKE ACTION

There has been an increasing interest in the preparation of thrombin for use as a haemostatic and for laboratory purposes during the past twenty years Seegers et al (1939) described the preparation of a highly active and relatively pure bovine thrombin and later defined a unit of activity thus being the amount of thrombin which will clot 1 c c of a standard fibrinogen solution in fifteen seconds Milstone (1941) and Adams and Taylor (1943) also studied methods for its preparation and purification An active human thrombin is prepared by an ether precipitation process by Keckwick and his co-workers at the Lister Institute London One of the earliest commercial thrombins of high potency was the interesting rabbit clotting globulin described by Parfentjev (1941a b) *The method of* separation which consists of ammonium sulphate precipitation of the globulin fraction of rabbit plasma would not be expected to produce a spontaneously active thrombin The precipitate does however develop a powerful thrombin activity and when in solution forms a stable and convenient preparation This material has been studied by Taylor et al (1941) and Lozner et al (1941)

Eagle (1937) has shown that the venom of some of the

rattlesnakes such as *Crotalus adamenteous Crotalus terrificus* and *Crotalus horridus* and of *Bothrops nummifera* have a thrombin-like action being able to coagulate fibrinogen directly and in the absence of calcium platelets or prothrombin Their coagulant action seems to vary directly as their proteolytic activity (Janszky 1950) Eagle (1937) and Eagle and Harris (1937) have studied the action of papain in producing coagulation It appears to act like the venoms already mentioned but coagulation only occurs within a limited range of concentration since digestion of fibrinogen may occur before coagulation if the concentration of the proteolytic enzyme is too high The theoretical implications of the thrombin-like action by proteolytic enzymes are of obvious interest and are discussed by Eagle

Other substances capable of coagulating fibrinogen are ninhydrin, alloxan and salicylaldehyde which were studied by Chargaff and Ziff (1941) and napthoquinone which has been extensively investigated by Lyons (1945a b) Lyons has based an individual theory of the thrombin-fibrinogen reaction on the ability of ninhydrin and napthoquinone to coagulate fibrinogen (Chapter II)

It should be realized that the coagulation produced by some of these enzymes and chemical substances does not resemble in all respects the normal fibrin clot Often the so-called coagulation consists of a gelatinous or ropey precipitate or even of a collection of flakes and particles so that it may not have much information to give on the normal process of fibrin formation Precipitation of fibrinogen can be achieved by protein precipitants of all kinds by heating or by increasing the salt concentration but such precipitates can hardly be regarded as related to the formation of fibrin by thrombin.

ACTIVATORS OF PROTHROMBIN

Many preparations having real or fancied thromboplastic activity have been produced for therapeutic and laboratory purposes Most of these are extracts of animal or human tissues prepared by methods so diverse that it would be out of place to consider them in detail here The most familiar is the dried acetone-treated brain preparation made from rabbit or human material which is used for the one-stage prothrombin time estimation of Quick. Other thromboplastins have included extracts of lung placenta, testis thymus platelets kidney and human milk. All these substances

behave as thromboplastins in that they require the presence of calcium and the various accelerators of thrombin generation in order to produce activation of prothrombin The species from which they are derived is an important consideration in assessing their action on human blood, as there is a well marked species-specificity (Chapter IV)

There are a number of other substances capable of inducing thrombin generation with or without the co-operation of calcium Douglas and Colebrook (1916) and Heard (1917a, b) found that trypsin hastened the clotting of blood an effect investigated by Mellanby (1935b) Eagle and Harris (1937) and Ferguson and Erikson (1939) Their results suggest that trypsin is capable of converting prothrombin into thrombin in the absence of calcium and as with papain there is a limited range of concentrations within which this effect is obtained Ferguson and Erikson (1939) showed that the effect of trypsin on prothrombin is increased by the presence of calcium and thromboplastin in the form of 'cephalin', and that coagulation times of sixteen seconds can be obtained by adding trypsin and calcium to citrated plasma They suggest that the action of trypsin is to mobilize an active thromboplastin from some inert precursor and they consider that a proteolytic factor in the plasma has a similar part in normal coagulation Intravenous injection of trypsin in animals can produce extensive intravascular coagulation and death within two minutes (Eagle 1937) Lesser amounts produce a lengthening of the clotting time as in the negative phase reaction with the appearance of an anticoagulant which is similar to but not identical with heparin (Quivy 1950)

THROMBOPLASTIN-LIKE VENOMS

A number of snake venoms act on prothrombin Eagle (1937) has shown that the venoms of *Notechis scutatus* (tiger snake) *Bothrops atrox* (fer-de-lance) and *Bothrops jararaca* have an action similar to that of trypsin since they are capable of producing thrombin from prothrombin even in the absence of calcium They have no effect on fibrinogen alone Russell s viper venom is particularly interesting Lamb (1903) observed that it was capable of clotting oxalated blood but in a comparatively feeble manner Macfarlane and Barnett (1934) showed that it actually had thromboplastin-like action of unparalleled power and required calcium for its full effect Using haeomophilic blood as an indicator it was

found that even when diluted many million times the coagulant action was still marked Macfarlane and Trevan (1936) found that this action was greatly increased by tissue extracts or lecithin and later (Macfarlane 1938a) it was established that the venom was incapable of clotting plasma from which all platelets and lipoid had been removed by high speed centrifuging filtration or extraction with lipoid solvents Its coagulant action was restored by the addition to such plasma of platelets or lecithin Leathes and Mellanby (1939) confirmed some of these observations and suggested that a venom-lipoid complex was analogous to a hypothetical enzyme-lipoid complex which might function as thromboplastin in physiological blood coagulation Macfarlane Trevan and Attwood (1941) further investigated this participation of a lipoid factor in coagulation and Macfarlane (1938a) observed that a coagulant with an action very similar to that of Russell s viper venom could be extracted from human saliva supporting the suggestion that the venom might be analogous to a natural clotting factor Recent work by Poole and Robinson (1956) suggests that the lipoid factor may be phosphatidyl ethanolamine

Experience with Russell s viper venom as a substitute for tissue thromboplastin in the performance of the one-stage prothrombin test suggests that it behaves differently from natural thromboplastin Its action is dependent upon the amount of available lipoid factors in the form of platelets free fat in the plasma or added lipoid In patients receiving dicoumarol or tromexan the prothrombin time may be considerably prolonged if brain thromboplastin is used but much less so if venom is used As it now seems established that the prolongation of the one-stage prothrombin time during treatment with dicoumarol and tromexan is probably due to a decrease in Factor VII it would appear that the action of the venom is less susceptible to a deficiency of Factor VII than is tissue thromboplastin

STAPHYLOCOAGULASE

Filtrates from pathogenic strains of *Staphyloccocus aureus* have been known to be capable of coagulating blood since the observations of Loeb (1903) The reaction was studied in detail by Walston (1935) and Smith and Hale (1944) Walston found that the filtrate ('coagulase') was capable of clotting his fibrinogen preparations and appeared therefore to be like thrombin in its action but unlike

thrombin it was not inhibited by heparin Smith and Hale (1944) on the other hand, observed that fibrinogen prepared by Mellanby's method was not coagulated by staphylocoagulase although it contained sufficient prothrombin to be coagulated by the addition of tissue extracts and calcium Oxalated plasma was rapidly coagulated and it was concluded that the plasma contained an activating factor which together with coagulase produced, in the absence of calcium a substance having the action of thrombin Duthie and Lorenz (1950) clarified to some extent these contradictions by finding that an inhibitor of coagulase activity might or might not be removed by the various processes used by previous authors so that failure to produce coagulation might not always indicate absence of the required activator They found that there was a parallel loss of activator and prothrombin during normal coagulation and prothrombin prepared from plasma by adsorbtion and elution contained the activator the supernatant contained Factor V but no activator Seitz filtration was supposed to remove prothrombin and the fact that activator was found to be present in the filtrate suggested at first sight that prothrombin cannot be the 'activator, though Duthie and Lorenz point out that this supposed loss of prothrombin by Seitz filtration may be more apparent than real (Chapter V) Tager and Lodge (1951) confirmed that the conversion of plasma to serum results in a loss of the coagulase activator factor and that the more effective the prothrombin conversion the greater this loss Seitz filtered plasma which has no demonstrable 'prothrombin activity retains the activating factor and these authors therefore assumed that prothrombin cannot be identical with activator But it must be emphasized that Seitz filtration may have unpredictable effects It has been observed by Koller et al (1951) that Seitz filtered plasma may actually contain considerable quantities of prothrombin though this prothrombin will not react with thromboplastin in the presence of Factor V and calcium since the required Factor VII has been removed by filtration The observations of Tager and Lodge (1951) and Duthie and Lorenz (1950) that Seitz filtered plasma contains the activator are not incompatible with a conclusion supported by other evidence that the activator and prothrombin are identical Recent work by Duthie and Lorenz (1952a b) supports this view since they have found a strict parallelism between the prothrombin levels estimated by a two-stage method and the amount of activator' present in the blood of patients receiving tromexan in liver disease

and in the prothrombin deficient patient described in Chapter XIV It is probable that staphylocoagulase reacts with prothrombin even in the absence of calcium and Factors V and VII to produce a form of thrombin This thrombin differs from normal thrombin since it is not inhibited by heparin nor by the antithrombin of the serum

HAEMOSTATICS

LOCAL HAEMOSTATICS

The control of prolonged or profuse bleeding is a problem that is constantly recurring in surgical and dental practice and to some extent even with the minor mishaps of everyday life If the mechanism of haemostasis is functioning normally nature is able to stop bleeding from the smaller vessels without artificial aid and in a comparatively short time though damage to larger vessels usually entails the use of ligatures or pressure to arrest the haemorrhage But when there is some defect in the haemostatic mechanism bleeding may continue almost indefinitely from even small injuries and this has stimulated the development of artificial haemostatic agents Some of these have already been described in the chapter on haemophilia

If the coagulation of the blood is reasonably efficient the provision of a large surface area of foreign material at the site of haemorrhage will stimulate fibrin formation and increase the solidity of the clot This is the basis of the traditional applications of cotton wool gauze and bandages to any injured area The cotton has a large area which stimulates blood coagulation and the threads become enmeshed in the newly formed fibrin thus providing a reinforcement which often produces a most effective haemostatic plug These measures effective when in conjunction with efficient clotting are more or less useless when there is a coagulation defect

In early attempts to control haemorrhage wounds were cauterized with red hot irons or dressed with tar acid alkalis copper sulphate ferric chloride and many other chemicals which precipitate or denature protein The object was to coagulate the bleeding surface so that an impervious layer of dead tissue would be formed through which bleeding could not occur Temporary haemostasis may have been achieved in these ways but the end result was usually disastrous because the destroyed tissue finally sloughed leaving an area larger than the original wound, from which renewed haemorrhage

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human and animal sources Seegers Warner Brinkhous and Smith (1939) described the use of a purified thrombin as a haemostatic agent in animal experiments Parfentjev (1941a b) described his rabbit clotting globulin which had a powerful thrombin action and was used as a local haemostatic by Lozner MacDonald Finland and Taylor (1941) and by van Creveld and Harmer (1941) It was found to be effective in controlling haemorrhage following tooth extraction in haemophilic subjects Seegers and Smith (1942) described the factors which influence the activity of thrombin preparations and other papers appeared describing the use of these thrombin preparations as local haemostatics (Adams and Taylor 1943 Tidrick Seegers and Warner 1943 and Stevenson 1944) In nose and throat surgery the dry powder can be applied directly to limited and accessible areas of oozing and in other sites a solution containing 20 000 units per ml can be sprayed by means of a syringe and fine needle into the required situation By these methods intranasal packing can often be avoided (Stevenson 1944) Thrombin was found to be valuable in controlling haemorrhage from skin graft sites by Cronkite Deaver and Lozner (1944) O'Connor (1945) injected 10 ml of thrombin solution containing 1000 units per ml into the bladder in cases of prostatectomy and Palomo (1945) confirmed the beneficial effects of this treatment stating that the absence of post-operative haemorrhage in such cases decreased the necessity for repeated irrigation Daly (1946) gave thrombin solution by mouth in an attempt to control haemorrhage in cases of gastro-intestinal bleeding He prevented the destruction of thrombin by gastric juice by giving phosphate buffer by mouth before the thrombin which was itself contained in phosphate buffer Thrombin has also been used in ophthalmic surgery by Parry and Laszlo (1946) and other applications are described by Seegers and Sharpe (1948)

ABSORBABLE DRESSINGS

The disadvantage of insoluble haemostatic dressings such as cotton wool or gauze is that though effective in arresting bleeding while in place they sooner or later have to be removed Removal usually entails a certain amount of tissue damage and a recurrence *of perhaps intractable haemorrhage in patients suffering* from a haemostatic defect The invention of dressings which are naturally absorbed in the course of time or which partially dissolve so that

occurred With increasing knowledge of the normal clotting mechanism it was realized that better results could be obtained by the application of substances supposed to hasten the clotting of blood in vitro Such things as gelatin pectin, horse serum and a number of commercial tissue preparations were used which though they had the great advantage of being comparatively harmless were almost inactive as coagulants (see Pickering 1928) Hirschfelder (1915) used a brain preparation as a local haemostatic in general surgery and it was also employed by Cecil (1917) in cases of prostatectomy and by Bastedo (1919) who gave it orally in cases of haematemesis Hanslick and Weidenthal (1919) administered cephalin in cases of haemophilia and of intestinal bleeding with reported benefit Other local applications of coagulant factors included dressings soaked in fresh normal human blood and proprietory preparations with a thrombin or thromboplastin-like action which were mostly of a comparatively low potency

Macfarlane and Barnett (1934) investigating the coagulant action of snake venoms observed the extraordinary potency of Russell's viper venom as a coagulant of haemophilic blood one of its characteristics being the fact that it is active when diluted to one in many millions Since any local haemostatic is liable to be rapidly diluted by issuing blood, its capacity to remain active in high dilution is of practical importance It was found possible to produce a haemostatic preparation which was both sterile and stable and this was used with success in a number of cases of accessible haemorrhage in haemophilic and other subjects (Macfarlane 1935 Cambrook 1936 Baker and Gibson 1936 Raimondi and Sangiovanni 1937 Hyatt and Buckland 1937 and others) The venom is capable of producing coagulation in therapeutic concentrations in from ten to twenty seconds and there is no indication that it causes delay of healing or increased liability to infection No ill effects following its use have been reported There is a good supply of the venom and proprietary preparations for local haemostatic use are available

Other snake venoms have also been used in a similar manner Rosenfeld and Lenke (1935) recommended the use of the venom of the Australian tiger snake and Peck Crimmins and Erf (1935) used fer-de-lance venom as a local haemostatic in haemophilia These venoms have not been used extensively

The development of large scale processes for the fractionation of blood and plasma has made available a supply of thrombin from

considerable advantage Further descriptions of its preparation were given by Bering (1944)

Glynn and Richards (1946) have investigated the antigenicity of fibrin foam prepared from bovine sources and have found that though in the fresh state this material is strongly antigenic and might therefore lead to allergic reactions in sensitive human subjects this antigenicity is lost after heat sterilization

An ingenious application of coagulation was made by Dees (1944) who injected plasma into the renal pelvis in cases of multiple renal calculi The resulting coagulum enclosed all the loose stones and could be removed together with these in one operation Reading (1946) has incorporated penicillin in a fibrin coagulum which can be introduced into infected cavities The slow diffusion of the penicillin from the clot results in a prolonged bacteriostatic action in the locality Seegers and Sharpe (1948) in a general review of these materials have discussed the question of possible allergy in human subjects as a result of the application of thrombin derived from animal sources They were unable to detect any sensitization in a series of forty-seven human subjects

Artificial Absorbable Dressings

The production of wool or gauze made from absorbable materials is a further advance In 1942 Yackel and Kenyon described the conversion of cellulose into an absorbable product by treatment with nitrogen dioxide The practical application in surgery of dressings made from oxidized cotton paper or gauze was discussed by Frantz (1943) Putnam (1943) combined soluble cellulose dressings with the use of thrombin as a haemostatic packing in brain surgery Seegers and Doub (1944) pointed out that oxidized cellulose might destroy thrombin unless the latter was dissolved in dilute sodium bicarbonate solution Uihlein and his co-workers (1945) made investigations to determine the length of time these dressings retained their structure in the tissues He found that after about 2½ days only a few recognizable fibres remained and that there was only a slight leucocytic reaction In 4½ days no recognizable cellulose fibres remained Lattes and Frantz (1945) evolved a test of absorbability by which the dressings could be standardized They considered that suitable material should be completely absorbed within fourteen days Frantz (1946) reviewing the advantages of oxidized cellulose as a haemostatic agent concluded that wounds are more

their fragments can be removed with the minimum of trauma is a considerable practical advance

Fibrin Preparations

Young and Medawar (1940) were apparently the first workers to use fibrin not it is true as a haemostatic but as a suture material in the repair of peripheral nerve injuries Seddon and Medawar (1942) described how plasma could be made to coagulate around the ends of severed human nerves forming a supporting structure in which nerve regeneration could take place The fibrin was absorbed by the natural processes of fibrinolysis and repair Tarlov and Benjamin (1942) improved this technique and observed that autologous plasma was less likely to produce inflammatory reactions than heterologus plasma Sano (1943) used a layer of the patient s plasma as a method of sticking down skin grafts The coagulation of the plasma produced a layer of fibrin which caused strong adhesion between the skin graft and the tissue to be grafted Macfarlane (1943) prepared films of fibrin which could be sterilized and kept for days or weeks and applied to raw areas such as burns being stuck down by a film of plasma or fibrinogen to which thrombin had been added These fibrin films formed an absorbable dressing which largely prevented the exudation of plasma and allowed healing to occur beneath them. They were also used in conjunction with thrombin or Russell s viper venom as coverings and plugging for tooth sockets in cases of haemophilia It was found that such absorbable dressings were preferable to gauze since removal was not required but unfortunately fibrinolysis was often so rapid that they were liable to disappear within a few hours A technical improvement was the fibrin foam described by Ingraham Bailey and Neelson (1944) This was produced as a dry sponge-like material which when moistened with saline became doughy and plastic so that it could be moulded to any form desired and used as an application to bleeding surfaces or cavities It was useful in neuro-surgery and preferable to muscle films or bone wax for haemostatic purposes Ingraham and Bailey (1944) found that fibrin films were useful in the repair of dural defects and prevented the formation of meningocerebral adhesions Bailey and Ingraham (1944) reviewed the use of fibrin in ninety-five neurological operations the foam being combined with a thrombin preparation and proving itself to be an effective haemostatic agent Bacteriostatic substances could be incorporated in this material with

produce a clot on the surface of a bleeding wound if haemorrhage continues beneath it Surface clots have little chance of producing a sufficient rise of pressure within the wound to stop bleeding because they cannot provide a water-tight covering of sufficient mechanical strength to withstand the blood pressure It is necessary to remove any loose clots from the bleeding surface and to apply the coagulant on gauze or preferably on an absorbable dressing The coagulant impregnated dressing is then pressed firmly on to the surface or into the cavity with a pressure sufficient to arrest the bleeding Blood on the surface will clot and adhere both to the tissue surface and to the haemostatic dressing The pressure should be applied for a period of time sufficient to ensure this local coagulation and adhesion at least five minutes being usually required Pressure can then be cautiously relaxed and it is usually found that the bleeding has been arrested The dressing must now be kept in place by some mechanical means which prevents it being dislodged, so that the clot sealing the damaged vessels is not broken In the case of a tooth socket some form of dental splint is usually required in the case of an external wound a firm pad and bandage is sufficient. It must be emphasized that the purpose of these mechanical aids is merely to keep the haemostatic dressing in place and they should not exert sufficient pressure to cut off the blood supply to the injured part since if this occurs healing will be delayed

In patients in whom the blood clotting mechanism is not seriously deranged such local applications are usually sufficient to produce permanent haemostasis Normally there is a more or less continual break-down of fibrin in wound cavities even without gross sepsis but new fibrin is formed by the coagulation of the slow exudate of blood or plasma which almost always occurs In haemophilia and other conditions in which coagulation is grossly deficient this replacement of fibrin will not occur unless there is continuous supply of some added coagulant Therefore in these cases the haemostatic application is effective for a limited period only varying from a few hours to a day depending on the degree of infection and inflammation. This destruction of the clot is particularly rapid in the mouth A tooth socket may be effectively filled with an absorbable dressing and coagulant with complete haemostasis but in about twelve hours the clot becomes digested and the whole haemostatic mass becomes loosened and ineffective Haemorrhage is then very likely to recur and will continue until the dressing is removed and the whole

easily packed with it than with ordinary gauze, since it is more pliable and becomes slightly sticky, and that packing is more efficient since the absorbable gauze swells slightly in contact with blood thereby filling the cavity completely The gauze forms an artificial clot which quickly produces haemostasis and if removal is necessary the mass is found to be sufficiently friable after forty-eight hours to be scraped away without danger of damaging the tissues and causing renewed bleeding Houser (1946) has used this material as a packing in controlling nasal bleeding and Fontain and Sammis (1948) have employed it as a packing for tooth sockets to control haemorrhage in thirty cases In all except three of these the material was absorbed satisfactorily

Another type of absorbable dressing is gelatin sponge which was described by Jenkins and Clarke (1945) This material also investigated by Correll and Wise (1945) is a foamy substance which is insoluble in water but absorbable by the tissues It has a haemostatic effect like that of oxidized cellulose and is non-antigenic Its use in neuro-surgery has been described by Abbott and Coleman (1946) and extensive tests in monkeys by Light and Prentice (1945) show that its implantation in the brain produces very little tissue reaction and that it is completely absorbed in from twenty to forty-five days Jenkins Janola and Clarke (1946) point out that the gelatin may be too rapidly destroyed presumably by proteolysis if there is an inflammatory reaction

Blaine (1946) introduced an absorbable calcium alginate which can be used as a dressing Oliver and Blaine (1950) describe its application as a haemostatic in neuro-surgery It is a convenient preparation easily sterilized by heat and its absorbability in the tissues can be controlled by altering its sodium content It appears to be an effective haemostatic

Lowry (1950) has experimented with a range of synthetic adhesives which form an absorbable haemostatic film on application to a bleeding surface He reports good results with a polyvinyl alcohol-sucrose-urea compound

GENERAL PRINCIPLES OF LOCAL HAEMOSTATIC APPLICATIONS

To be effective the application of any coagulant such as Russell's viper venom or thrombin must follow certain general principles The object is to produce a firm clot which extends down to and preferably into the orifices of any bleeding vessels It is useless to

of the blood following such administration in a variety of haemorrhagic states Wetterdal (19[illegible]6) used gelatin given sub-cutaneously in the treatment of haemorrhagic diseases of the newborn The fact that this treatment was combined with blood transfusion probably accounts for the beneficial results obtained which were ascribed to the gelatin There are also a number of proprietary haemostatics which are claimed to produce a general lowering of the blood coagulation time each of which has had its vogue Some of these are reviewed by Pickering (1928) The fact that few of them have survived to the present time strongly suggests that they really had very little beneficial effect It is only when some specific defect of the clotting mechanism can be corrected that an effective reduction of the clotting time can be produced by general means

SUMMARY TO CHAPTER XIX

1 Coagulants may be divided into two groups those which clot fibrinogen directly and those which promote thrombin formation The coagulants of fibrinogen include thrombin some snake venoms and various chemical substances The coagulants which favour thrombin formation include tissue extracts proteolytic enzymes some snake venoms and staphylocoagulase

2 Coagulants may be applied locally to control excessive bleeding Coagulants which have been used in this way are tissue extracts Russell s viper venom and thrombin. These coagulants are usually very effective if the blood coagulation system is normal but may be of temporary value only in haemophilia because the clot formed by the coagulants is removed by fibrinolysis and new clots do not form to reinforce the clots formed by the coagulant

3 Haemostasis and the healing of wounds may be favoured by the use of natural or artificial absorbable dressings

4 Measures to encourage a general increased coagulability of the blood are ineffective unless some specific defect is corrected

process repeated Provided that fresh applications are made with care to avoid tissue damage no harm will be done by repeated redressing, but it must be emphasized that anything which is liable to devitalize the tissues will delay the healing process and prolong the period during which haemorrhage is likely to occur

Temporary vasoconstriction produced by the application of adrenalin or cold in conjunction with a coagulant may assist in reducing haemorrhage and promoting the formation of a firm clot But these measures frequently lead to a reactionary vasodilatation which actually causes later haemorrhage so that they are best avoided

Protein precipitants of all sorts are worse than useless

GENERAL INCREASE IN THE COAGULABILITY OF THE BLOOD

The literature abounds with references to decreases in the clotting time produced in normal individuals and sometimes in haemophilic subjects by a bewildering variety of things given either by mouth or by injection Wright (1893) considered for instance that the administration of calcium decreased the clotting time and suggested that haemophilia should be treated by this means This claim was not of course substantiated but there still lingers a tendency to give patients calcium as a form of pre-medication in any surgical procedure likely to produce undue haemorrhage Vines (1920) observed that in one of the phases of anaphylactic shock there may be reduction of the clotting time (as opposed to the increased clotting time that usually occurs) and he advocated the treatment of haemophilia by a process of sensitization to horse serum followed by the deliberate induction of anaphylactic shock The desired changes in the clotting time that might occur under these conditions are too short-lived to be of practical value and the treatment obviously has grave inherent dangers of its own Neuhoff and Hirschfeld (1922) discovered that the administration of citrate shortened the clotting time and this observation has also led to various suggestions for the treatment of haemophilia Feissly (1925) investigated the action of pectin when injected intravenously in human subjects and animals and observed a reduction of the clotting time in all cases Many proprietary haemostatics have since included pectin though in actual practice very little material benefit seems to have been obtained Canterow (1926) recommended the injection of parathyroid extracts and claimed that there was an increased coagulability

that wounds which have ceased to bleed can be seen to be filled or covered with clotted blood from which it seems that the arrest of bleeding is the effect of coagulation The fact that any serious general defect of the clotting mechanism or any local condition which prevents clot formation is usually associated with prolonged bleeding clearly supports this view

Unfortunately closer examination of the problem reveals a number of facts which invalidate any such simple explanation of haemostasis These facts are provided by clinical observation of the haemorrhagic states and by the few available experiments

From clinical observations it is obvious that normal haemostasis often arrests bleeding from arterioles venules and capillaries in a few seconds although coagulation may take 5-10 minutes The mechanism by which bleeding from small stab wounds is arrested differs from that operating in the case of open wounds The determination of the time required for bleeding to stop from small stab wounds is known as the 'bleeding time test In conditions in which clotting is defective the bleeding time is usually normal but haemorrhage from open wounds continues for long periods In conditions in which the platelets are numerically or functionally deficient the bleeding time may be greatly prolonged, and there is also prolonged bleeding from open wounds In certain conditions such as von Willebrand s disease in which there is no demonstrable abnormality of the clotting mechanism or of the platelets prolonged bleeding both from small stab and open wounds occurs In other conditions such as pernicious anaemia, Banti s disease and in some thrombocytopenic cases after splenectomy low platelet counts may occur without abnormal haemorrhage No hypothesis of haemostasis is satisfactory unless it explains these clinical findings

There are very few reported investigations of the way in which clotting platelet agglutination and vascular function normally react together to arrest bleeding So much attention has been paid to the details of each of these components of the haemostatic mechanism that the larger and more general problem of haemostasis and its defects has been neglected

Hayem (1882) found that after an experimental wound made in the jugular vein of the dog had ceased to bleed the opening was filled by a mass of agglutinated platelets Kuttner and Baruch (1920) Magnus (1923) and Stegemann (1924a b) observed contraction of the arteries after injury Magnus (1924) and Macfar-

CHAPTER XX

THE SIGNIFICANCE OF BLOOD COAGULATION

INTRODUCTION

In the preceding chapters the component parts of the human blood coagulation mechanism and the faults which occur in various diseases have been presented in detail In this final chapter it is proposed to step back from the subject, to view the phenomenon of coagulation in perspective Unfortunately the clarity of this more general picture unlike that of some recent examples of the visual arts does not improve with distance Though details of the clotting system can now be portrayed with some precision the relationship of the system to other bodily processes is hazy and the observer is at once stimulated to ask questions What for instance is the real function of coagulation in the haemostatic mechanism? Is clotting concerned in the repair of wounds and resistance to infection? Has it any part in the metabolic or immunological reactions of the blood? Is it essential to life? And final *cri de cœur*, why does it have to be so complicated?

No book dealing exclusively with blood clotting and its disorders could be complete without an attempt to discuss these questions yet there are no satisfactory answers to any of them In some instances there is indirect evidence from which at least plausible hypotheses can be constructed in others only speculation is available Speculation has hitherto been avoided wherever possible now it can be avoided no longer Provided that it stimulates interest and that interest leads to experiment it serves a useful purpose

BLOOD COAGULATION AND HAEMOSTASIS

The most obvious function of coagulation is the control of bleeding The popular conception of the way in which it works is extremely simple blood issuing from damaged tissues solidifies into an impervious and adherent mass effectively preventing further haemorrhage This view is derived from the familiar observation

when the constricted vessels relaxed bleeding began and continued until it was stopped artificially or the animal was exsanguinated

H D Zucker (1949) studied by means of serial sections the histology of small puncture wounds in human skin which had been removed by biopsy soon after the wounds had ceased to bleed It was found that the damaged vessels (arterioles and venules) had been blocked by platelet masses Fibrin was not incorporated in any significant degree in these masses but was present in the wound tracks Only rarely were damaged capillaries seen and Zucker remarks that presumably recognition of these vessels is hindered by the traumatic distortion of their endothelium and possibly by collapse and by endothelial agglutination The few visible damaged capillaries were not sealed by platelets their open mouths seemed to be covered by fibrin In four cases of thrombocytopenic purpura eight punctures were examined and in all except two of these bleeding had ceased in five minutes or less and the wounds were filled with fibrin Platelet masses were absent In two punctures bleeding was continuing at the time of fixation of the specimen and though fibrin had formed the wounds also contained fluid blood

These different workers have each used their observation as a basis for speculations on the mechanism of haemostasis

Macfarlane (1941) suggested a two-stage mechanism He supposed that normally the blood flow from damaged small vessels is arrested by their temporary contraction and that during this period of haemostasis the blood lying in the wound has time to clot firmly On vascular re-dilation this adherent clot prevents a recurrence of the bleeding Abnormal bleeding occurs for two main reasons if vascular contraction is absent the bleeding from any injury even small stab wounds will be continuous since normal coagulation is thought to be incapable of arresting an actual flow of blood if there is a clotting defect bleeding will occur when the initial vascular contraction relaxes no firm clot having been formed in the interval (see Fig 50) In conditions associated with deficient vascular contraction the bleeding time will be prolonged In those in which clotting is deficient and vascular contraction normal there will be a normal bleeding time, since the period of active contraction will be sufficient for the edges of the small stab wound to adhere and dry up It is inferred that clotting is not essential for permanent haemostasis in such small wounds

This hypothesis seems to explain most of the observed facts in the

lane (1941) found that human capillaries disappeared from view after injury and the latter observer showed that this disappearance occurred even when the venous pressure was raised by 40 mm Hg and that the invisible capillaries normally reappeared again from 20 minutes to 2 hours after injury In thrombocytopenic purpura and von Willebrand s disease cutting of the capillaries did not cause their disappearance and continued haemorrhage occurred from the visible and damaged vessels M B Zucker (1947) studied the reactions to injury of the small vessels of the meso-appendix of the rat She found that there was no observable contraction of the non-muscular venules and that a plug of refractile material probably composed of agglutinated platelets formed at the tip of each severed vessel Vessels with muscular coats (presumably arterioles and small veins) contracted after injury and uninjured vessels in the vicinity also contracted This remote contraction occurred only when platelet plugs were formed at the site of injury In heparinized animals the platelet plugs either failed to form or were ineffective contraction was not observed and prolonged bleeding took place The local application of platelet extracts or of normal rat serum to uninjured vessels produced marked contraction Serum from thrombocytopenic animals had no constricting effect

Chen and Tsai (1948) studied the arrest of bleeding from small vessels in the frog and rabbit They observed that active contraction of the frog s web capillaries took place after injury but that mesenteric capillaries of the frog and rabbit did not contract In these non-contractile vessels pressure or cutting seemed to cause an adhesion of the endothelial walls which obliterated the lumen of the damaged vessel In the ear brain and mesenteric vessels of the rabbit it was observed that section of veins caused the formation of a refractile plug of platelets which became large enough to occlude the opening within three minutes Arteries and arterioles contracted actively after section and remained contracted for an hour or more Blood which escaped before contraction occurred clotted in situ during the period of contraction Occasionally rhythmic dilation and contraction of the injured vessels was observed and if this happened before coagulation was complete recurrence of bleeding took place Removal of the clot from the vessel mouth shortened the period of contraction In animals in which the coagulation time had been prolonged to two hours by the administration of dicoumarol the initial bleeding after injury was arrested by vaso-constriction but

can occur with a normal platelet count and a normal bleeding time with severe thrombocytopenia was accepted as evidence that the platelets were not essentially concerned in haemostasis The fact that thrombocytopenia is usually (but not always) associated with a prolongation of the bleeding time was explained by the observation that the platelets and the capillary endothelium are antigenically very similar and by supposing that any factor or toxic substance which damages the platelets causing thrombocytopenia is likely also to damage capillaries causing haemorrhage Recent observations on the occurrence of platelet lysins in the purpuras and the vascular damage produced by local application of agents producing thrombocytopenia which have already been discussed in Chapter XVI clearly support this supposition

M B Zucker (1947) though she observed contraction of the larger vessels of the rat s mesentery after injury considered that this was only a minor factor in haemostasis Small non muscular vessels did not contract but adhesion of their walls after puncture might prevent bleeding She considered that the most important haemostatic factor was the formation of platelet plugs The vaso-constricting effect of serum and platelet-extracts was noted but is not emphasized as being haemostatically important Fibrin formation is considered to be a minor factor in haemostasis since fibrin was not found in the platelet plugs The work was carried out on the vessels of the meso-appendix of the rat, and may therefore not be strictly applicable to the skin vessels of human beings The haemostatic mechanism has probably evolved as a defence against injury and the skin being the most frequently injured tissue may have developed defences which are different from those operating in tissues such as the mesentery which is not often traumatized

The disappointing feature of Zucker s interpretation of her findings is that it gives no explanation of the observed facts in the haemorrhagic states If as she supposes platelet plugs are the major haemostatic factor why does haemorrhage occur in the conditions with defective coagulation but normal platelets? If it is argued that because normal clotting may be related to platelet agglutination platelet plugs may not form if clotting is deficient why is the bleeding time normal in such conditions?

Chen and Tsai (1948) consider that the most important factor in the control of arterial or arteriolar bleeding is vascular contraction They also have put forward the idea of a two-stage haemostatic

haemorrhagic states The curious delayed haemorrhage so often observed in haemophilia is due to the period of vaso-constriction The demonstrable vascular abnormalities in the purpuras and in telangiectasia support the supposition that a functional vascular defect exists in these conditions Though it has been denied by some authors that capillaries can contract (e g Clark and Clark 1943) the

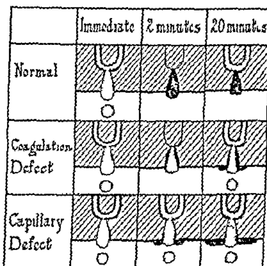


Fig 50 Diagram illustrating the suggested time relationship of normal capillary contraction redilatation and blood coagulation following injury and the two main defects of this mechanism which may occur A wound of the skin surface is shown in section injuring a capillary loop Fluid blood is represented by the dotted areas blood clot by solid black and the detached drops indicate active haemorrhage (Macfarlane 1945)

work of others seems to prove that they do in certain tissues Chen and Tsai (1948) have shown that capillary contractility varies from one animal to another and from one tissue to another The demonstration that non-muscular mesenteric vessels do not contract after injury (Zucker 1947) does not prove that skin vessels are incapable of contraction The work of Lewis (1923 19-4 1927) shows that human skin capillaries contract after suitable stimulation and are capable of exerting a considerable force in doing so Sanders Ebert and Florey (1940) have demonstrated convincingly active contraction of the capillaries of the rabbit s ear and showed that it is due to a rapid swelling of the endothelial cells

The weakness of the hypothesis is that it deliberately takes no account of the platelets The fact that a prolonged bleeding time

tion Fibrin formation has some part in maintaining haemostasis when vascular contraction relaxes and is concerned with the arrest of bleeding from the capillaries It is significant that no platelet plugs were seen occluding capillaries and that very few damaged capillaries were seen At least as many capillaries as arterioles and venules might be expected to be cut by a superficial puncture wound and the fact that they were not seen suggests that they were empty of blood A reasonable explanation is that at the time of fixation of the specimen the majority of the damaged capillaries were in a state of contraction and therefore invisible that a few had re-dilated but were plugged by the fibrin that formed during their contraction and that platelet plugs had not formed because bleeding from the capillaries had not persisted for any length of time

Zucker s view that fibrin formation is the principle factor in arresting capillary bleeding does not conform to the fact that the bleeding time test (which must involve bleeding from capillaries) is normal in those conditions in which fibrin formation is greatly delayed or even altogether absent

If all these observations are put together the following conclusions can be reached

(1) platelet masses form at the mouths of damaged vessels larger than capillaries

(2) after injury there is temporary contraction of these larger vessels and probably also of capillaries

(3) this vascular contraction may be stimulated by disintegrating platelets and it may fail to occur if the platelets are absent or prevented from agglutinating

(4) fibrin formation is not effective in arresting bleeding but is important for permanent haemostasis

It is possible that the mechanism of haemostasis thus depends on the summation of a number of factors which are not very closely integrated and which vary in relative importance with the type of injury and the size of the vessels injured The action of the platelets may be important in two ways The formation of platelet masses at the mouths of the larger vessels may be directly important in causing total blockage or in so impeding the blood flow that fibrin formation can occur and complete the sealing of the vessel The recent observations mentioned in Chapter XVI that platelets carry large amounts of 5-hydroxy tryptamine and adenosine triphosphate as well as adrenalin and histamine is directing attention to the local

mechanism the period of vascular contraction allowing firm clotting which prevents a renewal of the bleeding on re-dilation It was actually observed that, in dicoumarolized animals, bleeding which was temporarily arrested by vascular contraction recurred when dilation took place Bleeding from veins is thought to be arrested by the formation of platelet plugs in the case of venules both platelet plugging and adhesion of the walls are thought to be important Capillaries contracted on injury but the period of contraction was considered too short to be haemostatically effective The most important factor in the control of bleeding from the capillaries is supposed to be adhesion of the endothelial walls, presumably brought about by the pressure of the incising instrument

These suggestions involve four factors vascular contraction platelet agglutination endothelial adhesion and fibrin formation the importance of each varying with the type of vessel affected In small punctured wounds injuring venules arterioles and capillaries the important factors would be vascular contraction and endothelial adhesion Larger wounds would require platelet agglutination and fibrin formation in order that haemostasis should be efficient' This hypothesis explains most of the observed facts but introduces a new factor in the form of endothelial adhesion It is presumably inferred that adhesion is reduced in patients with a prolonged bleeding time It is difficult to see how such adhesion of capillary walls at the point of section could explain the disappearance of injured capillaries reported (and photographed) by Magnus and by Macfarlane unless it is supposed that the injured vessel fails to refill with blood or refills with transparent plasma

H. D Zucker (1949) considered from the results of his biopsy studies on human skin wounds that the formation of platelet plugs was the most important factor in the arrest of bleeding from all the vessels observed, except the capillaries He pictured these platelet masses as 'coffer-dams which aided by local vaso-constriction stop or slow the blood flow to the point where extravasated blood within the wound is given sufficient time to clot In the case of capillaries platelet plugs do not form and bleeding from them is arrested by fibrin formation aided by the low capillary blood-pressure and reflex contraction at the capillary mouths

Apparently therefore H D Zucker considers that platelet agglutination in the case of the larger vessels is the major factor in haemostasis with some ancillary assistance from vascular contrac-

nutritive material and at the same time a scaffolding for the upgrowth of blood vessels and fibrous tissue

There is some basis for the view that fibrin is a barrier to infection Menkin (1940) has shown that in inflamed tissues the spread of injected dyes and bacteria is greatly reduced as compared with their spread in normal tissues One of the features of the inflammatory process is an escape of fibrinogen (and presumably other coagulation factors) into the tissue spaces Clotting within these spaces then occurs and in severe inflammation the lymphatics draining the area become blocked by fibrin In this way there is formed a barrier relatively impervious to the passage of bacteria and large molecules which walls off the affected area If this fibrin barrier is broken down by the action of injected urea rapid spread of dyes and dissemination of bacteria can be demonstrated (Menkin 1940) It is possible that the rapid spreading of streptococcal infections may be due to the fibrinolysis produced by these organisms and that the localization of staphylococcal lesions is the result of increased fibrin formation stimulated by staphylocoagulase In clostridial myositis which is probably the most rapidly invasive infection occurring in man the absence of fibrin formation is a striking feature (Robb-Smith 1945) The way in which fibrin prevents the spread of infection is not clear The meshes of the fibrin clot are probably too loose to form a simple mechanical obstruction Possibly the large surface area of the fibrin has a powerful adsorbtive effect causing adhesion of bacteria and other particles

There is very little evidence to show that defects of coagulation increase the liability to infection In haemophilics there is no obviously greater incidence of spreading bacterial infections It is probable therefore that fibrin formation is only one of the localizing mechanisms existing in the tissues The invasiveness of organisms producing hyaluronidase suggests that the limiting effect of tissue hyaluronic acid is an important factor Animal experiments designed to show the effect of anticoagulants on the spread of infections might yield useful information

Evidence that fibrin is concerned in wound healing is almost non-existent The idea that fibrin network which fills a wound cavity forms a sort of scaffolding which guides fibroblasts and new capillaries is attractive but unproved It is tempting to visualize the growth of granulation tissue into a mass of fibrin which neatly fills the cavity to be obliterated, and which is lysed as the new tissue

vascular effects of these substances released during platelet breakdown It is probable, in fact that haemostasis may be only one of a series of local reactions to injury which are initiated by the blood It must be remembered, however, that in haemophilia and Christmas disease the bleeding time is typically normal despite the fact that platelet agglutination and breakdown is delayed Bigelow (1953) has actually *demonstrated that the release of 5-hydroxy tryptamine* in haemophilic blood is abnormally slow, so that the normal control of capillary bleeding in this condition remains obscure It is possible that the normal platelet agglutination observed in the blood of patients with congenital afibrinogenaemia might explain the comparative mildness of their haemorrhagic symptoms

There are other points concerning the haemostatic efficiency of coagulation which need to be clarified Clearly the whole-blood coagulation time is a poor index of the patient's liability to bleed in many cases of clinically severe haemophilia the clotting time may be almost within normal limits and hypoprothrombinaemia may cause severe bleeding without a significant lengthening of the clotting time Factors other than the time required to produce the first strands of fibrin must therefore be important Perhaps the tensile strength and density of the clot and its adhesion to the tissues are important No extensive investigation of these qualities and of their possible changes in disease has been carried out They may be related to the rate and duration of thrombin formation since changes in thromboplastin and thrombin generation seem to be more closely correlated with the liability to haemorrhage than is the simple clotting time

It is a depressing fact that in many cases of abnormal bleeding no defect of coagulation the platelets or of the vessels can be found demonstrating how little we know of the haemostatic mechanism

THE RELATION OF COAGULATION TO WOUND HEALING AND BACTERIAL INFECTION

It is generally supposed that in addition to its haemostatic function fibrin formation aids the repair of wounds and prevents the entry or spread of bacterial infection These ideas are set out in most text-books of pathology MacCullum (1940) for instance states that a blood clot is an effective protection against infection has a bactericidal power which prevents it becoming decomposed and forms a

ment activation and the initiation of haemolysis of the red cells Nolf (1922b) found that the red cells of dogs blood containing an autohaemolysin did not undergo haemolysis until the onset of coagulation Crosby and Dameshek (1950) have found that the Ac-globulin of Seegers is an important factor in producing haemolysis of the red cells in cases of paroxysmal nocturnal haemoglobinuria It is possible that the agglutination and lysis of the platelets which is an essential feature in the initiation of coagulation may be caused by the activation of a lytic factor which may also lyse red cells

COMPLEMENT

There have been a number of suggestions that coagulation and complement activity are in some way inter-related Both systems involve a series of components present in the plasma and there is evidence that the activation of complement may be related to the activation of the clotting system which might explain the observations on haemolysis quoted in the preceding paragraph In developing embryo chicks complement activity appears coincidentally with the coagulation factors (Pickering 1928) Many substances such as oxalate citrate fluoride heparin and soya-bean trypsin inhibitor antagonize both coagulation and complement activity Treatment of the plasma with zymin or ammonia or allowing it to age all destroy the complement activity of the plasma and greatly reduce its power to generate thrombin (Mann and Hurn 1948) The addition of a small proportion of serum restores both functions in each case suggesting that it is not prothrombin which is destroyed by these procedures In guinea-pigs maintained on a protein-deficient diet or given injections of carbon tetrachloride there is a parallel decrease of clotting efficiency and of complement titre suggesting that a factor common to both systems was reduced by these treatments (Rice Boulanger and Plummer 1951a, b) These workers were not able to identify the supposed common factor though they considered that evidence favoured the identity of complement mid-piece with prothrombin This was a suggestion which had already been put forward by Fuchs (1929) and refuted by Wising (1938) It is clear that the real relationship of complement to coagulation can only be determined by careful work in which there is full recognition of the difficulties of assaying prothrombin the various accelerators of prothrombin conversion and of the factors involved in the generation of intrinsic thromboplastin

forms The growth of such new tissue into a void with *no supporting* framework, and no guiding fibres is a more difficult concept

The suggestion that the blood clot has a nutritive value in the healing process seems to have little foundation It was considered by earlier workers that fibrin fibres were transformed into collagen but modern views on the structure of fibrin and collagen suggest that these two substances are so different that the conversion of one into the other is most unlikely Neither does it seem very probable that the products of lysed blood clots are metabolized in situ to be built up into new tissue But it is a fact that even trivial injuries in haemophilic subjects are often very slow to heal The literature abounds with reports of haemorrhage persisting for many days or weeks from some small cut or abrasion which would have healed completely in a few days in a normal subject In contrast it has been observed (Fraenkel and Honey 1955) that large wounds in a haemophilic patient healed very rapidly during the administration of animal antihaemophilic globulin It is possible therefore that the normal clotting mechanism or some part of it may be involved in the processes of repair

THE RELATION OF COAGULATION TO OTHER FUNCTIONS OF THE BLOOD

AGGLUTINATION AND HAEMOLYSIS

From time to time attempts have been made to link the observed processes of coagulation with other functions of the blood Hekma (1928) considered that fibrin formation was due to the agglutination of fibrinogen micellae by an agglutinin (thrombin) which is derived from cells or platelets and a plasma constituent Coagulation in his view apparently was a phenomenon analogous to or identical with the processes of erythrocyte or bacterial agglutination Pickering (1928) points out the fundamental dissimilarity of thrombin and the agglutinins which would appear to dispose of this theory Pickering himself (1928) considered that coagulation was merely one of the effects of a disturbance of the colloidal equilibrium of the plasma as a whole He visualized the plasma as a co-ordinated complex in which the less stable fractions (prothrombin and fibrinogen) are united to the more stable fractions (globulin and albumin) Disruption of this colloidal complex by the action of lysed platelets or tissue fluid results in coagulation and *also in such phenomena as comple-*

of the body have very little to support them It is certain that serious derangement of the efficiency of coagulation has serious consequences for the patient in the form of dangerous haemorrhage It is equally certain that blood coagulation in the sense of fibrin formation is not essential for survival A number of patients have now been studied in whom a total absence of fibrinogen precludes the possibility of fibrin formation as a significant factor in haemostasis or in any of the other processes in which it is supposed to be concerned It is true that these patients all suffered from a severe haemorrhagic diathesis yet some of them survived for many years with no greater disability than is produced by a mild form of haemophilia In these patients injuries ceased to bleed and healed bacterial infection was localized and protein metabolism proceeded all without the intervention of fibrin This does not mean that fibrin formation is not important in the normal person it does mean that the body can substitute other mechanisms for it if the need arises In afibrinogenaemic patients it may be significant that the rest of the clotting system may be relatively normal so that thrombin formation occurs in the usual way If thrombin is important in producing a vasoconstrictor substance from the platelets the normal thrombin production in afibrinogenaemia might lessen the severity of the haemorrhagic tendency

If available evidence tends to relegate fibrin formation to a rather subsidiary position among the physiological functions of the body it is by no means certain that the earlier stages of coagulation may not have other functions of unsuspected importance There are already hints that the production of local pain following injury to the tissues may be due to the development of a pain-producing factor in the blood after its contact with a foreign surface (Armstrong Jepson, Keele and Steward 1955) Miles and Wilhelm (1955) have described the appearance of a factor in serum which promotes increased capillary permeability the development of which may also be related to changes in the blood produced by contact. The physiological and pharmacological effects of platelets lysis which is promoted by the early reactions of the coagulation mechanism may be of great importance to the local and even general reactions to injury Thus the disproportion between the complexity of the clotting mechanism and the results it achieves may be more apparent than real because these results may entail more than the formation of fibrin. Even so some observers of the efforts of the coagulation research workers are convinced that all the various factors and inhibitors which are now

METABOLISM

Nolf (1908b) suggested that the blood clotting mechanism is normally concerned in protein metabolism and may therefore be regarded as the adaptation of a nutritive process as a defence against haemorrhage He considered that clotting of fibrinogen occurred more or less continuously in the circulating blood the fibrin being deposited on the vascular endothelium, and rapidly lysed by the normal fibrinolytic system The products of this protein breakdown are then used for protein synthesis The process of fibrin deposition and lysis almost certainly does occur in certain conditions an extreme example being the 'defibrination syndrome' observed in certain cases of abruptio placentae, and lobectomy (see Chapter XIII) It is not established however that a lesser degree of this process is a continuous normal function of the blood or even if it is that it serves any metabolic function Some of the evidence held to support this view can now be seen to be fallacious For instance the extremely rapid disappearance of fibrinogen from the blood after extirpation of the liver or following chloroform poisoning was taken to indicate a rapid normal consumption of fibrinogen revealed by a simple cessation of production It is probable that the precipitous fall of fibrinogen levels observed in these experiments is actually due to the intense fibrinolytic activity which such operative procedures almost certainly induce The true normal rate of fibrinogen consumption is not known The survival of transfused fibrinogen in a patient with congenital afibrinogenaemia was found to be 10-14 days by Pinniger and Prunty (1946) This if a valid index of the normal rate suggests a consumption of about 0 5-1 0 g of fibrinogen per day not an important fraction of the total protein turn-over of the body On the other hand patients with afibrinogenaemia might lack a normal ability to metabolize fibrinogen

THE COMPLEXITY OF THE COAGULATION SYSTEM

From the preceding sections it will be seen that our knowledge of the significance of the blood coagulation system is fragmentary Even its most obvious function the control of bleeding becomes ill-defined on examination suggestions that clotting may participate in such immune reactions as agglutination and complement activation or in wound healing and bacteriostasis or in the general metabolism

the fibrinolytic system with its marked similarity to the dual tissue and plasma factors of thromboplastin formation and the system of at least six enzymes and inhibitors concerned in the action of hyaluronidase on the tissues (hyaluronidase a and b anti-invasin I pro-invasin I anti-invasin II pro-invasin II anti-invasin III) as described by Haas (1946) to realize that other subsidiary systems have their own complications

The complexity of coagulation would be more easily understood no doubt, if a clear picture of the evolution of the process could be obtained Robb-Smith (1952) has pointed out that repair following abnormal tissue destruction is achieved by an acceleration of the normal processes of tissue maintenance and not by special processes only called into play on injury He therefore considers that blood coagulation is likely to be not a special defence mechanism but an adapted process of ordinary tissue activity As a part of the defence against trauma blood clotting is therefore likely to be a modified or adapted process that originally existed for purposes of current tissue activity Many of the more specialized biological processes are reflections of the internal functions of living cells in general The pouring of antibodies into the blood stream of an immunized individual for instance is an accentuated version of the internal formation of antibodies which takes place in any cell subjected to the action of an antigen Coagulation and liquefaction are two processes which occur in the cytoplasm of cells and enable them to transfer metabolic products extend pseudopodia and ingest particles (Monné 1948) and undergo mitotic division (Heilbrunn 1952) Heilbrunn (195-) has emphasized the similarity of protoplasmic coagulation to blood coagulation pointing out that both processes are inhibited by citrate oxalate and heparin and are stimulated by calcium, tissue fluids and injury It is possible therefore that the coagulation of the blood may have evolved from some internal cell mechanism required to produce the necessary rapid changes from sol to gel in the protoplasm Wooldridge (1893) considered that the plasma was a living medium analogous to the protoplasm of the cell, and it may be that various complex reactions which are limited to cellular protoplasm in lower animals may have developed into extra-cellular functions of the plasma in mammals Certainly in the lower animals and insects the part played by the cells in the phenomenon of coagulation is more obvious and important than in mammalian coagulation. Glavind (1947) states that many invertebrates have no coagula-

postulated are not merely unnecessary for clotting but in fact do not exist It is argued that they are artefacts produced by the crude methods of fractionation used by the research worker, fragments torn from a relatively simple and functionally active whole This view is really best expressed by Pickering (1928) who regarded the plasma as a single colloidal system It is, however, a view which gives no help to the research worker All systems even down to the atom have component parts and the intrinsic working of systems can only be studied by taking them to pieces From the point of view of outward function a system can be regarded as a unit When considering problems of transport, for instance one thinks of a motor car as a complete entity When considered as a mechanism the system has to be studied component by component in order to understand the way in which it functions and the faults to which it is liable But in this study the scale of the investigation must be kept in mind In the investigation of the mechanism of a car one obtains useful information by taking it to pieces each piece representing a mechanically functional component No additional information on the mechanical principles of the car would be obtained by further reducing these functional pieces to smaller fragments or subjecting them to chemical analysis One would learn something about metallurgy perhaps but not simple mechanics The same principle applies to blood clotting There is a limit to the useful information on the mechanism of coagulation obtainable by fractionation of the plasma but there is considerable evidence to show that this limit has not yet been reached All the factors described in this book appear to be real functional components of the clotting system They seem to be distinct entities not only because they can be separated in an active state but because the naturally occurring deficiency of each one results in a distinct disease process One must accept the idea that the clotting system really is *at least* as complex as modern work suggests and is probably far more complex than we now realize

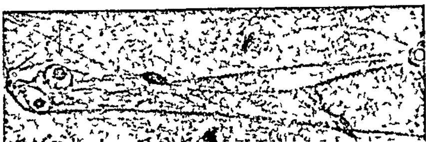
To consider this complexity as extraordinary is probably illogical It is likely that blood coagulation appears to be an unusually complex system only because it has been investigated with a vigour unusual in the study of biological processes of similar scope It is probable in fact that all biological processes however apparently simple on the surface will prove to be almost infinitely complex on persistent investigation One has only to consider what is already known about the complement system the red cell antigens and their antibodies

the fibrinolytic system with its marked similarity to the dual tissue and plasma factors of thromboplastin formation and the system of at least six enzymes and inhibitors concerned in the action of hyaluronidase on the tissues (hyaluronidase a and b anti-invasin I pro-invasin I anti-invasin II pro-invasin II anti-invasin III) as described by Haas (1946) to realize that other subsidiary systems have their own complications

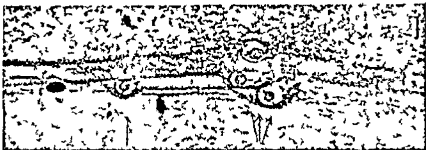
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tion of the blood in the usual sense but a clot is produced by agglutination of special cells In the crustacea there is coagulation of a fibrinogen-like substance, which requires calcium, and also a factor derived from the tissue cells which has some of the properties of thrombin and thromboplastin Gregoire (1951) studied the clotting mechanism of the haemolymph of many species of insects In those insects in which coagulation occurred two mechanisms could be distinguished usually occurring together but sometimes with one or other predominating in certain species One mechanism was the active protrusion of long pseudopodia by the haemocytes these linking up to form a cellular network (Plate 3) The resemblance to the less extensive linking of platelet pseudopodia observed in human blood by Budtz-Olsen (1951) is striking The other mechanism described by Gregoire is the formation of granular 'coagulation-islands in the immediate vicinity of the haemocytes These islands enlarge and coalesce and finally produce a solid coagulum sometimes with a veil-like quality suggesting fibre formation It seems clear that something is produced by the haemocytes which forms a precipitate in the surrounding haemolymph

The evolution of the complex plasma clotting system from this primitive and predominantly cellular type of clotting would be a fascinating study for any comparative physiologist with a knowledge of modern methods of investigating coagulation It is probable that much of the complexity of the mammalian system derives from the necessity to limit coagulation to the injured area and to guard against disastrous thrombosis of important vessels But this particular field remains almost completely unexplored In the human clotting system one can at present only observe the product of ages of evolutionary development and admire the endless resourcefulness with which Nature solves her problems



(a)



(b)

PLATE 3

(a) Coagulation in *Carcinus maenas*. Coagulum around three coagulocytes. From Grégoire and Florkin (1950).
(b) Coagulation in *Blaps gigas* showing coagulocytes and islands of coagulation. There is a deposition of granular particles along a system of parallel strings organized to form a meshwork. This meshwork probably arises both from cytoplasmic pseudopods and plasma substance. From Grégoire and Florkin (1950).

GLOSSARY OF TERMS

Accelerin (Owren 1950) A substance thought to be formed during clotting probably by the action of thrombin from pro-accelerin Pro-accelerin is synonymous with Factor V

Ac-globulin (Ware Guest and Seegers 1947b) Synonymous with Factor V

Activated Prothrombin (Quick and Stefanini 1949a) Prothrombin is thought to exist in plasma partly in a precursor form which requires activation before it can be converted to thrombin

Antiplasmin (Christensen and Macleod 1945) The natural inhibitor of the fibrinolytic enzyme plasmin

Antithrombin The natural inhibitor of thrombin In the blood thrombin is neutralized in various ways all of which are referred to as antithrombic

Antithromboplastin (Tocantins 1943) Thought to be a natural inhibitor for thromboplastin The term has not been defined in the light of work on the blood thromboplastin system

Cephalin In blood coagulation work cephalin is taken to be the fraction of some crude phospholipid (usually brain tissue) which is soluble in petroleum ether and ethyl ether and precipitated by ethyl alcohol It is not synonymous with phosphatidyl ethanolamine

Christmas Factor (Biggs et al 1952) Synonymous with plasma thromboplastin component Factor IX and antihaemophilic B factor A substance present in normal serum and lacking from the serum of patients with a haemophilia-like disease It is an essential component for blood thromboplastin formation

Christmas Disease (Biggs et al 1952) A condition closely resembling haemophilia which is associated with a reduction in the ability of the patient s serum to take part in the formation of blood thromboplastin The condition differs from haemophilia because mixtures of haemophilic and Christmas disease blood clot normally Synonymous with haemophilia B and P T C deficiency

Co-thromboplastin (Mann 1949 Mann and Hurn 1950) Probably synonymous with Factor VII But the phenomenon observed by the authors may also be encountered in Factor V deficiency

Convertin (Owren 1950a and b 1951) Thought to be formed from proconvertin (Factor VII) during clotting It is thought to be an intermediate product in the formation of prothrombinase from brain extract In many published experiments convertin may have been synonymous with prothrombinase

Cytozyme (Bordet 1913) A thromboplastic factor derived from platelets

Factor V (Owren 1947) A factor present in normal plasma which is not adsorbed by inorganic precipitates ($Al(OH)_3$ $BaSO_4$ etc) is consumed during clotting and is labile on storage It is necessary for the formation of prothrombinase from brain and for blood thromboplastin formation Its deficiency in plasma produces a long one-stage prothrombin time Synonymous with proaccelerin Ac-globulin prothrombin accelerator and the labile factor

Factor VI (Owren 1947) Thought to be a labile intermediate product in the formation of thromboplastin The exact significance of this factor is obscure and the term is no longer used

Factor VII (Koller et al 1951) A substance present in normal serum which is necessary for prothrombinase formation the deficiency of which produces a long one-stage prothrombin time Factor VII is deficient in the blood of patients treated with the dicoumarin drugs and in Vitamin K deficiency

Synonymous with proconvertin serum prothrombin conversion accelerator co-thromboplastin serum ac-globulin and serum accelerator

Factor VIII (Koller 1954) Synonymous with antihaemophilic globulin

Factor IX (Koller 1954) Synonymous with Christmas factor

Factor X (Koller 1954 and 1955) A factor said to be deficient from the blood of patients treated with dicoumarin drugs Its deficiency causes delayed blood thromboplastin formation The status of this factor is still unclear

Fibrinokinase (Astrup and Permin 1947 Astrup and Stage 1952) An activator of plasminogen derived from animal tissues which cannot be brought into aqueous solution by the usual procedures for extracting biologically active substances It can be obtained in solution by extraction with M Potassium thiocyanate

Fibrinolysin and Fibrinolysis General terms often used non-specifically for activity of the plasmin enzyme system

Haemophilia A Antihaemophilic globulin deficiency
Haemophilia B Christmas Disease
Heparin An anticoagulant derived from tissues thought to be localized in mast cells Heparin is a polysulphuric acid ester of a mucopolysaccharide i e a polysaccharide containing hexosamine
Heparin co-factor A substance present in the albumin fraction of plasma which is necessary for the anticoagulant action of purified heparin
Hypoprothrombinaemia A general term used to classify patients whose plasma gives a long one-stage prothrombin time This condition may be due to Factor V or Factor VII deficiency the presence of an inhibitor or fibrinogen deficiency it is very rarely due to true prothrombin deficiency
Labile factor (Quick 1943) Synonymous with Factor V
Metathrombin The name given to the inactive combination of thrombin and antithrombin
Parahaemophilia (Owren 1947) Synonymous with Factor V deficiency
Plasmin An active proteolytic enzyme derived from the globulin fraction of human or beef blood which has a high specificity for fibrin This specificity is thought to be due to the adsorbtion of plasmin by fibrin during clotting this adsorbtion separates plasmin from an inhibitory factor with which it is usually associated
Plasma Accelerator Globulin (Ware Guest and Seegers 1947a and b) Synonymous with Factor V
Plasma Thromboplastin Antecedent (Rosenthal 1954) Thought to be a component required for blood thromboplastin formation which is not adsorbed by inorganic precipitates and is present in normal serum Its deficiency is said to be the cause of a haemorrhagic diathesis resembling haemophilia The status of this factor is not clear at present the haemorrhagic tendency nominally associated with this factor is best considered as a syndrome of undetermined aetiology
Plasma Thromboplastin Component (Aggeler et al 1952) Synonymous with Christmas factor
Platelet co-factor I (Seegers 1954) Synonymous with antihaemophilic globulin
Platelet co-factor II (Seegers 1954) Synonymous with the Christmas factor

Convertin (Owren 1950a and b, 1951) Thought to be formed from proconvertin (Factor VII) during clotting It is thought to be an intermediate product in the formation of prothrombinase from brain extract In many published experiments convertin may have been synonymous with prothrombinase

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Fibrinolysin and Fibrinolysis General terms often used non-specifically for activity of the plasmin enzyme system

stance is ill-defined The phenomena for which the term was devised may have been due to Factor VII activity or to intermediate products of thromboplastin formation

Serum Prothrombin Conversion Accelerator *S P C A* (Alexander and co-workers 1949) Synonymous with Factor VII

Staphylocoagulase A comparatively heat stable substance produced by staphylococci which in the presence of a heat labile factor present in human plasma and certain animal plasmas and tissue extracts will cause plasma to clot

Staphylokinase A substance produced by staphylococci which can activate plasminogen to plasmin under suitable conditions It is probably similar to but not necessarily identical with streptokinase

Threone (Seegers 1954) A name for a theoretical intermediate product in the formation of blood thromboplastin thought to be derived from platelet co-factor I (antihaemophilic globulin) and platelets

Thromb-Elastograph (Hartert 1951) A machine for studying the increased rigidity of blood or plasma during clotting

Thromb-Elastogramme (Hartert 1951) A tracing obtained with a thromb-elastograph

Thromboasthenia We use this term to define a condition where grossly abnormal giant platelets are found in the blood These platelets are usually functionally abnormal releasing reduced amounts of blood thromboplastin when used in the thromboplastin generation test

Thrombocatalysin (Lengenhagger 1946) An enzyme factor derived from blood which may or may not be the same as plasmin which was thought to activate a precursor of thromboplastin (prothrombokinase) This work has not been confirmed or disproved

Thrombocytolysin (Brinkhous 1947) A plasma factor which was thought to be activated by contact with a foreign surface which caused lysis of platelets and liberation of thromboplastin Brinkhous experiments suggest a deficiency of this substance in haemophilic blood From its supposed properties and mode of action it resembles the Christmas factor

Thrombogen (Morawitz 1905) Synonymous with prothrombin Nolf (1908 1928) has also used this term for the factor now referred to as Factor V

Proaccelerin (Owren 1950) Synonymous with Factor V
Proserozyme (Bordet 1920) A theoretical precursor of prothrombin Not generally accepted at any time
Prothrombin The only essential precursor of thrombin
Prothrombin A
Quick (1943) Prothrombin A was a labile factor now called the labile factor and synonymous with Factor V
Quick (1947) a component of prothrombin not the labile factor
Quick and Stefanini (1949a) An inactive precursor of prothrombin now usually referred to as inactive prothrombin This latter view is similar to that of Bordet (1920)
Munro and Munro (1947) *True prothrombin*
Prothrombin B
(Munro and Munro 1947) Probably synonymous with Factor V
Quick (1943 1947) True prothrombin the immediate precursor of thrombin (see also prothrombin A)
Prothrombin accelerator (Fantl and Nance 1948) Synonymous with Factor V
Prothrombin conversion accelerator PCA (Owen and Bollman 1948) Synonymous with Factor VII
Prothrombinase (Owren Rapaport Hjort and Aas 1954) The direct activator of prothrombin derived from the reaction between brain extract Factor V Factor VII and Calcium
Prothrombokinase (Collingwood and MacMahon 1912) A theoretical precursor of thromboplastin thought to be derived from platelets
Prothrombokinin (Lengenhagger 1946) A theoretical precursor of blood thromboplastin it cannot be identified with any of the precursors recognized today
Pseudohaemophilia A term used rather variously for clotting defects resembling haemophilia Not infrequently the term is used for patients who have circulating anticoagulants in their blood
Serozyme (Bordet and Delange 1912 Bordet 1920) An activated form of prothrombin
Serum Accelerator (Jacox 1949) Probably synonymous with Factor VII but the observed *phenomenon may also have been influenced* by labile intermediate products in the formation of blood thromboplastin and by blood thromboplastin itself
Serum Accelerator Globulin (Ware and Seegers 1948b) This sub-

APPENDIX II

THE SYSTEMATIC INVESTIGATION OF COAGULATION DEFECTS

This appendix is intended as a rough practical guide to the laboratory diagnosis of clotting defects The first step in diagnosis is an inquiry into the clinical and family history This will determine the severity of the condition and by this inquiry a number of patients referred to the laboratory may be excluded For example patients who have undergone tonsillectomy and several dental extractions with no excessive bleeding will not be found to have haemophilia Having taken the history certain preliminary tests should be made These are

One-stage Prothrombin time	Appendix IV 7
Whole blood clotting time	Appendix IV 1
Platelet count	Appendix IV 6
Bleeding time	Appendix IV 4
Tourniquet test	Appendix IV 5

Having obtained the results of these tests a preliminary subdivision of the patients into three groups A B and C (according to the results) is possible The worker should read these headings and decide which one applies to the patient in question In each group additional tests lead to a more sharply defined diagnosis

We have included some short practical notes in this section All of the subject matter of these notes is available in other parts of the book, but it was thought that it might be easier for the worker new to the subject to have this information readily at hand

It should be realized that this guide includes defects that have been described clearly in the literature It is quite possible that patients may be encountered who do not fit into any of these categories With these patients ingenuity in the use of established tests and the invention of new techniques may advance knowledge

Thrombokinase Synonymous with thromboplastin

Thrombokinin (Lengenhagger 1946) A term used for a blood thromboplastin theoretically derived from a precursor (prothrombokinin) by the action of a proteolytic enzyme (thrombocatalysin)

Thromboplastin This term used to be used to designate a direct activator of prothrombin and the substance was thought to be derived from tissue extracts or platelets It is now recognized that tissue extracts do not activate prothrombin directly In this book the term is used in a non-specific sense for any substance which shortens the calcium clotting time of normal plasma The direct activator (or activators) of prothrombin when finally identified must then be given new terms (see prothrombinase)

Thromboplastinogen (Quick 1947) A precursor of blood thromboplastin deficient in haemophilic blood The term is thus synonymous with antihaemophilic globulin

Thrombozyme (Nolf 1908 1928) Probably synonymous with prothrombin as used by Nolf

von Willebrand s Disease A bleeding tendency inherited as a simple dominant in which the capillaries fail to contract normally on injury The patients tend to suffer from oozing from small lesions and a long bleeding time is found

injured Inquiry about the bleeding following dental extractions or tonsillectomy will often disclose the disease In our experience patients who have the mildest degree of haemophilia usually bleed for 1-2 weeks after dental extraction and have always required transfusion after tonsillectomy

Results of other Laboratory Tests

The whole blood clotting time may be normal or prolonged In our experience 9 of 10 male patients with a grossly prolonged clotting time have haemophilia A normal result is of no significance The prothrombin consumption test is usually abnormal but normal or doubtful results are not uncommon The antihaemophilic globulin assay method usually records no antihaemophilic globulin The mildly affected patients usually have 5-30 per cent by this method the normal range by this method is 50-200 per cent

2 *Mixtures 3 and 4 give abnormal results mixtures 1 and 2 give normal results*

Diagnosis — *Christmas Disease*

The mode of inheritance and clinical features of Christmas disease are identical with those of haemophilia In our experience about 1 of 10 patients with these clinical findings have Christmas disease Among the cases that we have seen more of the Christmas disease patients have been mildly affected their blood giving normal results with the clotting time and often also with the prothrombin consumption test

3 *Mixtures 2 3 and 4 give abnormal results*

(*a*) The presence of *a circulating anticoagulant* should be suspected and tests for inhibitor carried out (Appendix IV 24)

Notes on patients with circulating anticoagulants

A proportion of the patients who have circulating anticoagulants also have haemophilia or Christmas disease in these cases the history of bleeding will date from infancy and the patients will be males In other cases the anticoagulant is acquired in adult life and a sudden recent onset of bleeding tendency will be evident males or females may be affected The patients may or may not have some associated disease in a proportion of the female patients the anticoagulant follows within a year of delivery

A *ONE-STAGE PROTHOMBIN TIME PLATELET COUNT, BLEEDING TIME AND TOURNIQUET TEST ARE NORMAL THE CLOTTING TIME IS NORMAL OR PROLONGED*

Having obtained these results the thromboplastin generation test (Appendix IV, 28) should be carried out using mixtures of reagents as indicated below and further subdivision is possible from the results of this test

Mixture	*Source of $Al(OH)_3$ treated plasma*	*Source of serum*	*Source of Platelets or Lipoid*
1	Normal	Normal	Normal
2	Patient	Normal	Normal
3	Normal	Patient	Normal
4	Patient	Patient	Normal

1 *Mixtures 2 and 4 give abnormal results 1 and 3 give normal results*
Diagnosis – *Haemophilia*

Notes on Haemophilia

Inheritance

About half of the patients give a history of similar bleeding in other members of the family The family history should be taken in detail and recorded diagrammatically Particular questions being asked about the patient s brothers the patient s sister s sons the patient s mother s brothers and the patient s mother s sister s sons Even when the first question elicits the answer no one else in my family has any bleeding tendency the history should nevertheless be taken in detail Many patients who give a negative history have very small families

Clinical Features of Bleeding Tendency

The clinical severity of haemophilia varies very greatly from one family to another In the severely affected patient the history is characteristic massive haematomata develop after trivial injury haemarthroses appear soon after the patient starts to walk apparently spontaneous bleeding occurs from other sites epistaxis haematuria and gastro-intestinal bleeding are all common At the other extreme are patients who have no trouble unless they are quite severely

5 Platelet count usually above 100 000 cu mm Platelets very large Bleeding time and/or tourniquet test usually abnormal Clot retraction and prothrombin consumption may be abnormal Clot retraction and prothrombin consumption usually abnormal unless the platelet count is greatly raised Platelets may give abnormal results when used to replace normal platelets in the thromboplastin generation test Other members of the family may or may not be affected

Diagnosis — *Thromboasthenia (Functional Platelet Deficiency)*

C *ONE-STAGE PROTHROMBIN TIME ABNORMAL BLEEDING TIME TOURNIQUET TEST AND PLATELET COUNT NORMAL*

1 Whole blood clotting time indefinitely prolonged No clot with added thrombin Very small precipitate on 25 per cent saturation of plasma with $(NH_4)_2SO_4$

Diagnosis — *Afibrinogenaemia*

2 One-stage prothrombin time considerably shortened by the addition of 10 per cent of normal plasma treated with $Al(OH)_3$ or by the same proportion of plasma from a patient treated with one of the dicoumarin group of drugs Shortening of 2-3 seconds has no significance (Appendix IV 10 and 11)

Diagnosis — *Factor V Deficiency*

Note on Factor V Deficiency

Whole blood clotting time usually long Prothrombin consumption test usually abnormal One-stage prothrombin time not shortened by the addition of normal serum or purified Factor VII Two-stage prothrombin test normal Thromboplastin generation test will probably give abnormal results if the patient's $Al(OH)_3$ treated plasma is substituted for the normal reagents and the patient's platelets are used to replace normal platelets (Normal platelets have some adsorbed Factor V)

3 One-stage Prothrombin time *not* shortened by 10 per cent of Al(OH) treated plasma or by the plasma of patients treated with the dicoumarin drugs One-stage prothrombin time greatly short-

(*b*) No circulating anticoagulant A combined deficiency of antihaemophilic globulin and Christmas factor should be suspected

4 *Mixtures 1 2 and 3 give normal results but mixture 4 is abnormal*
This pattern of results is said to be characteristic of *Rosenthal's Syndrome (P T A deficiency)*

Notes on Rosenthal s Syndrome
Clinically patients in this group resemble the mildly affected haemophilia patients The condition has a dominant inheritance and thus females are affected as often as males We have seen about six families with mild symptoms and dominant inheritance but the results of the thromboplastin generation test have tended to vary

B *ONE-STAGE PROTHOMBIN TIME NORMAL BLEEDING TIME TOURNIQUET TEST OR PLATELET COUNT ABNORMAL*

1 Platelet count at some stage below 100 000 cu mm Tourniquet test and bleeding time abnormal Prothrombin consumption deficient Onset recent no replacement of bone marrow by abnormal tissue No history of taking drugs

Diagnosis — *Essential Thrombocytopenic Purpura*

2 Same as B 1 but a history of taking drugs particularly sedormid, gold sulphonamide quinine phenacetin barbiturates The appropriate drug lyses or agglutinates sensitized platelets (Ackroyd 1949)

Diagnosis — *Toxic Thrombocytopenic Purpura*

3 Same as B 1 but the marrow is replaced by leukaemic or other tissue or normal bone marrow absent

Diagnosis — *Secondary Thrombocytopenic Purpura*

4 Platelet count always above 100 000 cu mm Platelet morphology normal Tourniquet test positive or negative Bleeding time prolonged Clot retraction normal Prothrombin consumption may be slightly abnormal Level of antihaemophilic globulin may be reduced Capillaries microscopically abnormal Inherited as a simple dominant

Diagnosis — *von Willebrand s Disease*

5 Platelet count usually above 100,000 cu.mm. Platelets very large. Bleeding time and/or tourniquet test usually abnormal. Clot retraction and prothrombin consumption may be abnormal. Clot retraction and prothrombin consumption usually abnormal unless the platelet count is greatly raised. Platelets may give abnormal results when used to replace normal platelets in the thromboplastin generation test. Other members of the family may or may not be affected.

Diagnosis — *Thrombasthenia (Functional Platelet Deficiency)*

C. *ONE-STAGE PROTHROMBIN TIME ABNORMAL. BLEEDING TIME, TOURNIQUET TEST AND PLATELET COUNT NORMAL*

1 Whole blood clotting time indefinitely prolonged. No clot with added thrombin. Very small precipitate on 25 per cent saturation of plasma with $(NH_4)_2SO_4$

Diagnosis — *Afibrinogenaemia*

2. One-stage prothrombin time considerably shortened by the addition of 10 per cent of normal plasma treated with $Al(OH)_3$ or by the same proportion of plasma from a patient treated with one of the dicoumarin group of drugs. Shortening of 2-3 seconds has no significance. (Appendix IV 10 and 11)

Diagnosis — *Factor V Deficiency*

Note on Factor V Deficiency

Whole blood clotting time usually long. Prothrombin consumption test usually abnormal. One-stage prothrombin time not shortened by the addition of normal serum or purified Factor VII. Two-stage prothrombin test normal. Thromboplastin generation test will probably give abnormal results if the patient's $Al(OH)_3$ treated plasma is substituted for the normal reagents and the patient's platelets are used to replace normal platelets (Normal platelets have some adsorbed Factor V)

3 One-stage Prothrombin time *not* shortened by 10 per cent of $Al(OH)_3$ treated plasma or by the plasma of patients treated with the dicoumarin drugs. One-stage prothrombin time greatly shor-

(b) No circulating anticoagulant A combined deficiency of antihaemophilic globulin and Christmas factor should be suspected.

4 *Mixtures 1 2 and 3 give normal results but mixture 4 is abnormal*
This pattern of results is said to be characteristic of *Rosenthal's Syndrome (P T A deficiency)*

Notes on Rosenthal's Syndrome
Clinically patients in this group resemble the mildly affected haemophilia patients The condition has a dominant inheritance and thus females are affected as often as males We have seen about six families with mild symptoms and dominant inheritance but the results of the thromboplastin generation test have tended to vary

B *ONE-STAGE PROTHOMBIN TIME NORMAL BLEEDING TIME TOURNIQUET TEST OR PLATELET COUNT ABNORMAL*

1 Platelet count at some stage below 100 000 cu mm. Tourniquet test and bleeding time abnormal Prothrombin consumption deficient Onset recent no replacement of bone marrow by abnormal tissue No history of taking drugs

Diagnosis — *Essential Thrombocytopenic Purpura*

2 Same as B 1 but a history of taking drugs particularly sedormid gold, sulphonamide quinine phenacetin barbiturates The appropriate drug lyses or agglutinates sensitized platelets (Ackroyd 1949)

Diagnosis — *Toxic Thrombocytopenic Purpura*

3 Same as B 1 but the marrow is replaced by leukaemic or other tissue or normal bone marrow absent

Diagnosis — *Secondary Thrombocytopenic Purpura*

4 Platelet count always above 100 000 cu.mm Platelet morphology normal Tourniquet test positive or negative Bleeding time prolonged Clot retraction normal Prothrombin consumption may be slightly abnormal Level of antihaemophilic globulin may be reduced. Capillaries microscopically abnormal. Inherited as a simple dominant

Diagnosis — *von Willebrand's Disease*

5 Factor V Factor VII and Prothrombin all normal Less than 100 mg /ml fibrinogen present in the blood

Diagnosis — *Fibrinopenia*

6 One-stage prothrombin time shortened by toluidine blue or protamine sulphate (Appendix IV 23) Lee and White clotting time prolonged

Diagnosis — *Heparinaemia*

7 One-stage prothrombin time of normal plasma lengthened by the addition of 10-50 per cent of the patient s plasma Tests unaffected by toluidine blue or protamine sulphate (Appendix IV 26)

Diagnosis — *Inhibitor*

ened by 10 per cent of normal serum Two-stage prothrombin test normal Thromboplastin generation test normal Level of A H G and Christmas factor normal (Appendix IV, 12 and 13)

Diagnosis — *Factor VII Deficiency of the Hicks-Jurgens type*

Note on the Study of Factor VII Deficiency

A study of patients whose blood has a long one-stage prothrombin time which is shortened by the addition of normal serum has revealed several types of defect

(*a*) Pure Factor VII deficiency (Hicks-Jurgens see Chapter XIV)

(*b*) Combined Factor VII deficiency and a serum thromboplastin factor deficiency which does not appear to be of Christmas factor Bergsagel (1955) de Vries (1955)

(*c*) A combined Factor VII and prothrombin deficiency and a serum thromboplastin factor deficiency which may be of the Christmas factor (Newcombe 1955)

No general rule for the detailed study of these patients can be laid down Each case will repay very careful study Tests which should be done with all cases include a study of various pathological sera to determine which will shorten the one-stage prothrombin time of the patient s plasma Serum samples of particular interest are those from patients with haemophilia Christmas disease and patients treated with the dicoumarin anticoagulants In addition the two-stage prothrombin test should be carried out in all cases The thromboplastin generation test should also be done and mixtures of various pathological sera should be tested

It should be noted that the defect (c) above may respond well to transfusion therapy the abnormality being controlled in one case by 500 ml of fresh plasma once in 4 weeks

4 One-stage prothrombin time not shortened by $Al(OH)_3$ treated plasma or by normal serum Two-stage test gives grossly abnormal results (Appendix IV 14)

Diagnosis — *Prothrombin Deficiency*

Note on Prothrombin Deficiency

Congenital or idiopathic prothrombin deficiency is very rare In the form secondary to liver disease or Vitamin K deficiency quite severe prothrombin deficiency may cause little lengthening of the one-stage prothrombin time the two-stage test should therefore always be done

6 *Glyoxaline (Imidazole) Buffer (Mertz and Owen 1940)*

A solution of base is prepared by dissolving 680 mg of glyoxaline (Light s Organic Chemicals) in 50 ml of distilled water The buffer at pH 7 3 is made by mixing 2 5 parts of base with 1 86 parts of 0 1 N HCl and 5 64 parts of distilled water

7 *Owen s Buffer*

11 75 gm of sodium diethylbarbiturate and 14 67 gm NaCl are dissolved in a mixture of 1570 ml of distilled water and 430 ml of 0 1 N HCl The pH should be 7 35

8 *Buffered Saline for Fibrinolysin Test*

Veronal-acetate base solution for preparation of Michaelis (1931) buffers

9 714 gm sodium acetate trihydrate and 14 714 gm sodium diethylbarbiturate are dissolved together in carbon dioxide-free distilled water and the solution made up to 500 ml This constitutes the buffer base

To make buffered saline of pH 7 4±0 05 (Glass electrode) 5 ml of this base solution are mixed with 5 ml of 0 1 N HCl and 90 ml of 0 85 per cent NaCl

9 *Al(OH)$_3$ for adsorbtion (Bertho and Grassman 1938)*

100 ml of ammonia solution (s g 0 88 diluted one in two) are poured into 600 ml of water at 63 C containing 22 gm of ammonium sulphate and the temperature is rapidly brought to 58 C The mixture is stirred vigorously and poured rapidly in one lot into a solution of 76 7 gm of ammonium alum at a temperature of 58 C in 1000 ml of distilled water The temperature rises to 61 C Stir for 10 minutes without letting the temperature fall below 58 C and then separate the precipitate by centrifuging The precipitate is washed five times with 1500 ml of distilled water separating the precipitate by centrifuging on each occasion To the first washing water is added 0 22 ml of s g 0 88 ammonia and to the second 0 44 ml before the washing is started The whole procedure should not take much more than five hours The precipitate is suspended in the least amount of water that is required to make a gelatinous suspension that can be pipetted Usually the addition of water giving a final volume to about 700 ml produces a suitable consistency The

APPENDIX III

THE PREPARATION OF REAGENTS AND COAGULATION FACTORS

A REAGENTS

1 *Calcium Chloride*

A 2 per cent solution of anhydrous calcium chloride is made up and the concentration is determined by titration The solution is then suitably diluted to give an M/10 solution (11 1 gm per 1000 ml) From this M/20 and M/40 can be made as desired

2 *Sodium Citrate*

3 1 3 8 or 5 per cent solutions are made by dissolving the appropriate weight of trisodium citrate ($Na_3C_6H_5O_7,2H_2O$) in distilled water The solutions should be dispensed in 2 oz bottles and sterilized in the autoclave to prevent the rapid growth of contaminating bacteria and moulds

3 *Sodium Oxalate*

A 1 34 per cent (w/v) solution is used

4 *Phosphate Buffer for the Preparation of Fibrinogen (Modified from Jaques 1943)*

A 2M phosphate buffer at pH 6 6 is made by dissolving 817 gm of anhydrous KH_2PO_4 in 1000 ml of distilled water to which has been added 750 ml of 4N KOH (168 gm) After warming to dissolve the volume is made up to 3000 ml and the solution is filtered M and M/4 solutions are made from this stock solution

5 *Phosphate Buffer for the Elution of Prothrombin*

Two solutions are prepared as follows

(*a*) 11 88 gm of $Na_2HPO_4.2H_2O$ are dissolved in 1000 ml of water

(*b*) 9 1 gm of KH_2PO_4 are dissolved in 1000 ml of water

To make a buffer of pH 8 9 7 parts of solution A are mixed with 0 3 parts of solution B

6 *Glyoxaline (Imidazole) Buffer (Mertz and Owen 1940)*
A solution of base is prepared by dissolving 680 mg of glyoxaline (Light s Organic Chemicals) in 50 ml of distilled water The buffer at pH 7 3 is made by mixing 2 5 parts of base with 1 86 parts of 0 1 N HCl and 5 64 parts of distilled water

7 *Owen s Buffer*
11 75 gm of sodium diethylbarbiturate and 14 67 gm NaCl are dissolved in a mixture of 1570 ml of distilled water and 430 ml of 0 1 N HCl The pH should be 7 35

8 *Buffered Saline for Fibrinolysin Test*
Veronal-acetate base solution for preparation of Michaelis (1931) buffers
9 714 gm sodium acetate trihydrate and 14 714 gm sodium diethylbarbiturate are dissolved together in carbon dioxide-free distilled water and the solution made up to 500 ml This constitutes the buffer base
To make buffered saline of pH 7.4±0 05 (Glass electrode) 5 ml of this base solution are mixed with 5 ml of 0 1 N HCl and 90 ml of 0 85 per cent NaCl

9 *Al(OH) for adsorbtion (Bertho and Grassman 1938)*
100 ml of ammonia solution (s g 0 88 diluted one in two) are poured into 600 ml of water at 63 C containing 22 gm. of ammonium sulphate and the temperature is rapidly brought to 58 C The mixture is stirred vigorously and poured rapidly in one lot into a solution of 76 7 gm of ammonium alum at a temperature of 58 C in 1000 ml of distilled water The temperature rises to 61 C Stir for 10 minutes without letting the temperature fall below 58 C and then separate the precipitate by centrifuging The precipitate is washed five times with 1500 ml of distilled water separating the precipitate by centrifuging on each occasion To the first washing water is added 0 22 ml of s g 0 88 ammonia and to the second 0 44 ml before the washing is started The whole procedure should not take much more than five hours The precipitate is suspended in the least amount of water that is required to make a gelatinous suspension that can be pipetted Usually the addition of water giving a final volume to about 700 ml produces a suitable consistency The

preparation keeps for about six weeks The addition of 1-4 ml of this suspension to 100 ml of plasma is usually sufficient to remove most of prothrombin and Factor VII present Smaller amounts can be prepared by using proportional quantities of reagents

A preparation made by British Drug Houses can also be used by suspending 1 gm of the alumina gel in 4 ml of distilled water

10 *$BaSO_4$ for adsorbtion*

1 lb of X-ray $BaSO_4$ is suspended in about 4000 ml of 0 005 M trisodium citrate and allowed to stand overnight The milky supernatant is drawn off and discarded The sediment is resuspended in a further 4000 ml of citrate solution and again allowed to settle The sediment is then collected on any conveniently substantial filter paper and dried in a hot air oven at 100 C The dried material is then powdered This washing is required because the $BaSO_4$ contains some very fine material which is not removed from plasma by centrifuging at 2000 r p m

11 *Solutions of Toluidine Blue*

Solutions of toluidine blue in 0 85 per cent saline are prepared to contain 50 mg and 25 mg per cent of toluidine blue

12 *Cleaning and Preparation of Glassware*

All obvious clots should be removed from glass tubes when the tests are completed Dried clots are difficult to remove The tubes should then be washed soaked in dilute hydrochloric acid and rinsed with several changes of tap water and distilled water The tubes are then dried in a hot air oven

13 *Preparation of Silicone Glassware*

The silicone D C 1107 of the Midland Silicones Ltd can be used This preparation is used dissolved as a 5 per cent (v/v) solution in carbon tetrachloride The cleaned glassware is filled with the solution which is emptied back into the bottle and any excess allowed to drain off The glass is then baked in a dry sterilizing oven for ½-1 hours at 160°-210° C The coating of silicone and baking may have to be repeated to obtain a very good surface

Alternatively the preparations Drifilm or Desicote may be used these do not require to be baked

B COAGULATION FACTORS

1 *Collection of Blood*

(a) *Citrated Plasma*

Whole blood is collected into a dry paraffined syringe and mixed with sodium citrate in the proportion of nine parts of blood to one part of citrate solution in a graduated centrifuge tube To obtain platelet rich plasma the blood is centrifuged for 5 minutes at 1500 r p m To obtain platelet poor plasma the blood is centrifuged at 3000 r p m. for 30 minutes or at 15 000 r p m for 5 minutes

(b) Oxalated plasma is prepared similarly after mixing 9 parts of blood with 1 part of 1 34 per cent sodium oxalate

(c) *Serum*

For the thromboplastin generation test or for the preparation of coagulation factors serum should contain a minimum amount of prothrombin and intermediate products of clotting To facilitate prothrombin conversion 2 or 3 glass beads are dropped into the whole blood and the tube inverted gently during clotting When clotting is complete the tube is incubated for at least four hours at 37 C Complete removal of intermediate products probably requires incubation at 37° C for 24 hours but for the diagnosis of Christmas disease it is usually found that 4 hours incubation is sufficient

2 *Fibrinogen*

To 1000 ml of discarded blood bank plasma is added 50 ml of Al(OH) suspension The mixture is stirred at room temperature for 15 minutes The mixture is centrifuged and the sediment discarded The supernatant plasma is tested by the one-stage prothrombin time method and the clotting time should exceed one minute If a shorter clotting time is obtained the absorbtion with Al(OH) should be repeated The plasma is cooled to about 12-14° C and to this is added an equal volume of 2M phosphate buffer which has been previously cooled to 2-4 C in the refrigerator the mixture is stood for 15 minutes to allow the formation of a precipitate The mixture is centrifuged for 10 minutes at 2000 r p m and the supernatant is discarded The precipitate is washed with 1000 ml of M phosphate buffer and the precipitate recovered by centrifuging The precipitate is then dissolved in 500 ml of M/4 phosphate buffer The volume is measured and the fibrinogen reprecipitated by adding an equal

volume of 2M phosphate buffer This precipitate is sedimented by centrifuging washed with 500 ml of M phosphate buffer and redissolved in 500 ml of M/4 phosphate buffer The precipitation and washing are repeated once more and the final precipitate is dissolved in 100-150 ml of citrate saline (9 parts of 0 85 per cent saline and 1 part of 3 8 per cent trisodium citrate) The solution is dialysed in the cold overnight against 5000 ml of citrate saline the dialysing fluid must be stirred during this time The product is centrifuged to remove any precipitate that may have formed and freeze dried Drying is completed in a vacuum desiccator over P_2O_5 The dried product is weighed and for use portions are weighed out and dissolved in distilled water to reconstitute the original solution For example if the total yield of dried material was 2 gm and this was dissolved originally in 100 ml the appropriate concentration would be 20 mg/ml This solution is too concentrated for use in most clotting tests, it is further diluted 1 in 2 to 1 in 5 with 0 85 saline for use The best concentration is determined by trial and error for a particular batch The solution should be the least concentration which will give a firm solid clot on the addition of thrombin *Solutions which give fine wispy clots or flakes of fibrin are too weak.*

If it is not proposed to freeze dry the product smaller amounts e g 100 ml of plasma may be used and the final precipitate dissolved in 50 ml of citrate saline

3 *Prothrombin*

(a) *Using $Al(OH)_3$* Sufficient $Al(OH)_3$ suspension (usually about 0 5 ml) is added to 10 ml of fresh normal citrated platelet poor plasma to lengthen the one-stage prothrombin time test to 1 minute The mixture is incubated at 37° C for 30 minutes and the precipitate sedimented by centrifuging The precipitate is washed twice with cold distilled water and the prothrombin is eluted by mixing the sediment with 5-10 ml of phosphate buffer at pH8 The mixture is allowed to stand at 37° C for one hour The $Al(OH)_3$ is deposited by centrifuging and the supernatant which now contains the prothrombin is adjusted to pH 7 with 2 per cent acetic acid and dialysed overnight in the cold against stirred citrate saline (9 parts of 0 85 per cent saline and one part of 3 8 per cent trisodium citrate)

(b) *Using $BaSO_4$* 1 gm of washed $BaSO_4$ (see Appendix III A 10) is added to 10 ml of fresh normal platelet poor oxalated plasma It should be noted that oxalated plasma *must* be used because $BaSO_4$ will

not adsorb in the presence of citrate The $BaSO_4$ is mixed with the plasma and the mixture allowed to stand at 37° C for 15 minutes The $BaSO_4$ is sedimented by centrifuging at 2000 r p m for 20 minutes The supernatant is discarded and the sediment washed twice with cold distilled water The prothrombin is eluted from the precipitate by adding 5 ml of 5 per cent trisodium citrate The mixture with citrate is allowed to stand for 15 minutes at 37° C The inactive $BaSO_4$ is deposited by centrifuging and the supernatant containing the prothrombin is dialysed overnight in the cold against 5000 ml of stirred citrate saline (9 parts of 0 85 per cent NaCl and one part of 3 8 per cent trisodium citrate) The solution may be frozen at -20 C if it is not to be used immediately Much activity is usually lost on freeze drying

Two methods of preparing prothrombin are given Sometimes it is found that the prothrombin prepared with $Al(OH)_3$ is contaminated with antithrombin On the other hand the $Al(OH)_3$ method has the advantage that it can be used on discarded bank plasma large quantities of which are easily obtainable

4 *Antihaemophilic Globulin*

The $Al(OH)_3$ treated plasma which remains after the preparation of prothrombin is used (see 3a above) The supernatant which contains little prothrombin Factor VII or Christmas factor is precipitated at 33 per cent saturation with $(NH_4)_2SO_4$ For example if 10 ml of $Al(OH)_3$ treated plasma is used 5 ml of saturated $(NH_4)_2SO_4$ is added The precipitate is sedimented by centrifuging and dissolved in a volume of 0 85 per cent saline equal to one-half of that of the original $Al(OH)_3$ treated plasma used (in the example thus would be 5 ml) The solution is dialysed overnight in the cold against 5000 ml of stirred citrate saline (9 parts of 0 85 per cent saline and one part of 3 8 per cent trisodium citrate) The volume is measured and made up to that of the original plasma (10 ml in the example) Antihaemophilic globulin can also be prepared in the same way from the supernatant plasma after $BaSO_4$ adsorbtion (see 3b above) The material made in this way is of low activity but is usually sufficiently active for experimental purposes

5 *Factor V*

Factor V may be made from the supernatant from the preparation of antihaemophilic globulin. The $(NH_4)_2SO_4$ saturation of the

solution is raised to 50 per cent by adding $\frac{1}{3}$ of the volume of saturated $(NH_4)_2SO_4$ The precipitate which forms is sedimented by centrifuging it is found that a relatively high centrifugal force is required (3000 r p m for 30 minutes or 15 000 r p m for 5 minutes) The precipitate is dissolved in 0 85 per cent saline equal to half of the original plasma volume (5 ml in the example) and dialysed overnight in the cold against 5000 ml of stirred citrate saline (9 parts of 0 85 per cent saline and 1 part of 3 8 per cent trisodium citrate) The volume is measured and made up to that of the original plasma (10 ml in the example)

In our experience the product derived from $BaSO_4$ treated oxalated plasma has a higher Factor V activity than that obtained from the $Al(OH)_3$ treated citrated plasma

6 *Thrombin*

Parke Davis or Maws Thrombin Topical may be used These preparations are made from bovine plasma but appear to behave with human fibrinogen in exactly the same manner as human thrombin Human thrombin may be made from citrated bank plasma as follows

100 ml of plasma are diluted to 1000 ml with distilled water The pH is adjusted to 5 3 with 2 per cent acetic acid The precipitate is dissolved in 25 ml of 0 85 per cent saline and the pH adjusted to 7 with 2 per cent Na_2CO_3 3 ml of 0 25 M $CaCl_2$ are added and the coagulated fibrinogen removed as it forms After standing for 2 hours for full thrombin formation the crude solution is purified by adding one volume of acetone to one volume of thrombin at room temperature The precipitate is separated by centrifuging The thrombin is extracted from the precipitate with 25 ml of 0 85 per cent saline The saline extract is centrifuged and precipitate discarded The solution of thrombin obtained in this way may have a strength of about one-fifth of the most concentrated solution of thrombin topical (1000 units per ml)

7 *Factor VII*

10 ml of serum are adsorbed with $BaSO_4$ (100 mg/ml of serum) for 15 minutes at 37° C The $BaSO_4$ and adsorbed proteins are sedimented by centrifuging The deposit is washed twice with cold distilled water and the final deposit is treated with 5 ml of a solution of 5 per cent trisodium citrate in 0 85 per cent NaCl for 15 minutes

at 37° C The inactive $BaSO_4$ is removed by centrifuging and the supernatant is dialysed overnight in the cold against 5000 ml of stirred citrate saline (9 parts of 0 85 per cent saline and 1 part of 3 8 per cent trisodium citrate) After dialysis the volume is measured and made up to 10 ml The product prepared in this way will have both Factor VII and Christmas factor activity

8 *Factor VII deficient ox plasma for the measurement of Factor VII activity*

Oxalated ox blood is collected in the slaughter house by mixing 9 parts of blood and 1 part of 2 5 per cent potassium oxalate (monohydrate) The plasma is separated by centrifuging and stored overnight at 5 C It is then filtered slowly through a 30 per cent asbestos seitz filter using 1 pad for every 15 ml of plasma The plasma is stored frozen solid in small amounts so that samples used are not refrozen The method usually removes all of the Factor VII but leaves 30 per cent of residual prothrombin The product is tested using the assay mixture (see Appendix IV 13)

0 1 ml of each of the following in 3 separate tests
(a) normal serum diluted 1/10 with glyoxaline buffer pH 7 3 or
(b) a similar dilution of plasma or (c) saline
0 1 seitz filtered ox plasma.
0 1 ml brain extract
0 1 M/40 CaCl

The mixture should clot in 25-30 seconds with plasma or serum but the clotting time should exceed 5 minutes with saline replacing plasma or serum If the clotting time in the presence of serum is long then too much prothrombin has been removed If the saline control is short too much Factor VII remains

9 *Christmas Factor by the Method of White Aggeler and Glendenning* (1953)

Whole blood is allowed to clot and the clot allowed to stand for 24 hours at room temperature The serum is separated and 9 volumes of serum are mixed with 1 volume of 3 2 per cent trisodium citrate This mixture is allowed to stand for 30 minutes at 37° C The pH is adjusted to 2 9 by dropwise addition of N HCl and the acidified serum is stood for 2 hours at 37° C This procedure destroys most of the Factor VII activity The pH is then returned to neutral

solution is raised to 50 per cent by adding ⅓ of the volume of saturated $(NH_4)_2SO_4$. The precipitate which forms is sedimented by centrifuging it is found that a relatively high centrifugal force is required (3000 r p m for 30 minutes or 15 000 r p m for 5 minutes) The precipitate is dissolved in 0 85 per cent saline equal to half of the original plasma volume (5 ml in the example) and dialysed overnight in the cold against 5000 ml of stirred citrate saline (9 parts of 0 85 per cent saline and 1 part of 3 8 per cent trisodium citrate) The volume is measured and made up to that of the original plasma (10 ml in the example)

In our experience the product derived from $BaSO_4$ treated oxalated plasma has a higher Factor V activity than that obtained from the $Al(OH)_3$ treated citrated plasma

6 *Thrombin*

Parke Davis or Maws Thrombin Topical may be used These preparations are made from bovine plasma but appear to behave with human fibrinogen in exactly the same manner as human thrombin Human thrombin may be made from citrated bank plasma as follows

100 ml of plasma are diluted to 1000 ml with distilled water The pH is adjusted to 5 3 with 2 per cent acetic acid The precipitate is dissolved in 25 ml of 0 85 per cent saline and the pH adjusted to 7 with 2 per cent Na_2CO_3 3 ml of 0 25 M $CaCl_2$ are added and the coagulated fibrinogen removed as it forms After standing for 2 hours for full thrombin formation the crude solution is purified by adding one volume of acetone to one volume of thrombin at room temperature The precipitate is separated by centrifuging The thrombin is extracted from the precipitate with 25 ml of 0 85 per cent saline The saline extract is centrifuged and precipitate discarded The solution of thrombin obtained in this way may have a strength of about one-fifth of the most concentrated solution of thrombin topical (1000 units per ml)

7 *Factor VII*

10 ml of serum are adsorbed with $BaSO_4$ (100 mg/ml of serum) for 15 minutes at 37° C The $BaSO_4$ and adsorbed proteins are sedimented by centrifuging The deposit is washed twice with cold distilled water and the final deposit is treated with 5 ml of a solution of 5 per cent trisodium citrate in 0 85 per cent NaCl for 15 minutes

(b) Preparation of thromboplastin suspension for use 0 5 gm of dried material is suspended in 10 ml of 0 85 per cent saline and the mixture is incubated at 37° C for 15 minutes The suspension is mixed once or twice during this period and the coarse particles are then allowed to settle by gravity

12 *Saline extract of Brain for use in the Factor VII assay method and in the prothrombin-proconvertion (p and p) method*

A human brain is freed from blood vessels and meninges and washed The whole brain is macerated for about 2 minutes with warm 0 85 per cent saline in a Waring-blender For a whole brain 1500 ml of saline heated to 37 C are used The emulsion is centrifuged for 25 minutes at 2000 r p m and the sediment is discarded The supernatant is tested by the one-stage prothrombin time test undiluted and diluted 1 in 2 1 in 3 and 1 in 5 with 0 85 per cent saline The dilution which gives the minimum clotting time is selected. The whole mixture is diluted appropriately and 10 per cent of Owren s buffer is added The final fluid is dispensed into containers which will provide a day s supply and stored frozen solid at −20 C

13 *Preparation of Phospholipid for the Antihaemophilic globulin assay method (Folch 1942 Hays and Lein 1945)*

A whole normal human brain freed from meninges and blood vessels is washed and macerated in a Waring-blender The weight is determined and the volume (usually 1000–1300 ml) is measured The mush is extracted with three lots of acetone each 4 times the volume of the original brain tissue The extracted material is filtered on a suction pump and the precipitate dried at 37 C overnight The precipitate is extracted with a mixture of 2 volumes of petroleum ether boiling point <40 C and 3 volumes boiling point 40–60 C The total volume of petroleum ether used is twice that of the original brain mush The petroleum ether extraction is repeated Between each extraction the ether is filtered through a solvent extracted filter paper The two extracts are combined and evaporated to dryness in vacuo at 45 C The remaining solid is dissolved in 1/5 of the original volume of ethyl ether The solution is left in the cold until a deposit forms This is removed by centrifuging The clear solution is evaporated in vacuo to about 100 ml /kg of original mush Leave in the cold for a precipitate to form

with N NaOH The treated serum is then adsorbed with $BaSO_4$ (100 mg/ml of serum) at 37° C for 15 minutes The $BaSO_4$ is sedimented by centrifuging and washed twice with cold distilled water After again centrifuging the $BaSO_4$ is treated with 5 per cent trisodium citrate to elute the Christmas factor The most convenient amount of citrate to use is one-half of the original serum volume (for 10 ml of serum 5 ml of citrate are used) The citrate eluate is dialysed overnight in the cold against 5000 ml of stirred citrate saline (9 parts 0 85 per cent saline and 1 part 3 8 per cent trisodium citrate) and the volume of the dialysed fluid made up to that of the original serum

10 *Platelet suspension*

Platelet rich plasma (Appendix III B 1) is centrifuged at 3000 r p m for 30 minutes or at 15 000 r p m for 5 minutes The platelets are deposited as a small button at the bottom of the tube The clear plasma is removed and may be used as substrate for the thromboplastin generation test The platelets are suspended in 0 85 per cent saline and deposited by centrifuging The platelets are washed once more with 0 85 per cent saline After depositing they are suspended in 0 85 per cent saline to $\frac{1}{2}$-$\frac{1}{3}$ of the original plasma volume If 10 ml of platelet rich plasma was used the platelets will be resuspended in 3-5 ml of 0 85 per cent saline For the thromboplastin generation test it will often be found that a further $\frac{1}{2}$ dilution is possible but it is wise in the first instance to prepare a more concentrated suspension The suspension often keeps quite well for 1-2 weeks if frozen solid at −15 to −20° C Suspensions which are thawed and then refrozen frequently lose much activity

11 *Brain Thromboplastin for use in the one-stage prothrombin time test*

(*a*) Dried brain powder Fresh human brain is collected in the post-mortem room and all superficial vessels and meninges are removed The substance is macerated with three to four times its volume of acetone in a mortar The acetone is replaced four times and the granular powder is dried on a suction filter It is unnecessary and undesirable to reduce the material to a fine powder because when a fine powder is produced the particles tend to rise to the top when the substance is suspended in saline The dried brain can be stored in bottles at room temperature with no special precautions with little deterioration for periods up to three months

(*b*) Preparation of thromboplastin suspension for use 0 5 gm of dried material is suspended in 10 ml of 0 85 per cent saline and the mixture is incubated at 37° C for 15 minutes The suspension is mixed once or twice during this period and the coarse particles are then allowed to settle by gravity

12 *Saline extract of Brain for use in the Factor VII assay method and in the prothrombin-proconvertion (p and p) method*

A human brain is freed from blood vessels and meninges and washed The whole brain is macerated for about 2 minutes with warm 0 85 per cent saline in a Waring-blender For a whole brain 1500 ml of saline heated to 37 C are used The emulsion is centrifuged for 25 minutes at 2000 r p m and the sediment is discarded The supernatant is tested by the one-stage prothrombin time test undiluted and diluted 1 in 2 1 in 3 and 1 in 5 with 0 85 per cent saline The dilution which gives the minimum clotting time is selected. The whole mixture is diluted appropriately and 10 per cent of Owren s buffer is added The final fluid is dispensed into containers which will provide a day s supply and stored frozen solid at −20 C

13 *Preparation of Phospholipid for the Antihaemophilic globulin assay method (Folch 1942 Hays and Lein 1945)*

A whole normal human brain freed from meninges and blood vessels is washed and macerated in a Waring-blender The weight is determined and the volume (usually 1000-1300 ml) is measured The mush is extracted with three lots of acetone each 4 times the volume of the original brain tissue The extracted material is filtered on a suction pump and the precipitate dried at 37° C overnight The precipitate is extracted with a mixture of 2 volumes of petroleum ether boiling point <40 C and 3 volumes boiling point 40-60 C The total volume of petroleum ether used is twice that of the original brain mush The petroleum ether extraction is repeated Between each extraction the ether is filtered through a solvent extracted filter paper The two extracts are combined and evaporated to dryness in vacuo at 45 C The remaining solid is dissolved in 1/5 of the original volume of ethyl ether The solution is left in the cold until a deposit forms This is removed by centrifuging The clear solution is evaporated in vacuo to about 100 ml/kg of original mush Leave in the cold for a precipitate to form

(12-24 hours) which is removed by centrifuging and discarded The solution is treated with 5 volumes of acetone and the precipitate is collected by centrifuging dried and stored in a dark bottle in a vacuum desiccator

For use 125 mg of this crude phospholipid are suspended in 5 ml of distilled water, the mixture being shaken and stirred until the emulsion is uniform The suspension is then diluted with 0 85 per cent saline to give a suspension of optimum concentration for the test This varies from 1/10-1/1000 and must be determined by experiment The diluted emulsion may be frozen solid when its activity is maintained for about 6 weeks

14 *Chloroform Extract of Brain for the Thromboplastin Generation Test (Bell and Alton* 1954)

0 5-1 gm of acetone dried brain are washed with 20 ml of acetone and the mixture left for 2 hours The brain tissue is recovered by centrifuging and the supernatant discarded The sedimented brain powder is dried on a water pump and the powder extracted for 2 hours with 20 ml of chloroform During this time the mixture must be shaken After this the suspension is filtered and the solid material discarded The filtrate is dried in vacuo and the resulting lipid is suspended in 5-10 ml of 0 85 per cent saline The saline emulsion may be diluted to about 1/100 for use The optimum dilution must be determined by trial in the test

This lipoid may be satisfactory for use in the antihaemophilic globulin assay method but it may give too short clotting times in mixtures which do not contain antihaemophilic globulin

TECHNICAL METHODS

1 WHOLE BLOOD CLOTTING TIME

(*a*) Method of Lee and White (1913)

The test is carried out in clean dry glass test tubes measuring 2½ in × ⅜ in The tubes must be perfectly clean and absolutely dry Venous blood is collected in a dry paraffined syringe The needles should be very sharp and of size 18-19 gauge The needle should enter the vein directly and any difficulty in obtaining the blood invalidates the test 1 ml of blood is placed in each of the four small tubes at 37 ° C and the tubes are tilted in turn at about ½-minute intervals until each tube can be inverted without spilling the blood As the blood begins to enter the syringe the stop-watch is started and the clotting time is recorded from this time until the blood has solidified in the tube The clotting time of each tube is recorded separately and the average of the four tests is reported By this method the clotting time of normal blood usually lies between 5 and 10 minutes

It is important to use standard apparatus and standard technique for this test because variations may influence both the clotting time and the prothrombin consumption test which is carried out on the blood collected for the Lee and White clotting time test For example if the tubes are rinsed with saline before use or if the tubes are stoppered the results will be different Similarly if a system is adopted in which one tube is tilted until coagulation occurs and then the second third and fourth are tilted in series and then the clotting time for the fourth sample will be longer than in the standard test The normal range for the Lee and White clotting time test differs in different laboratories and even from time to time in the same laboratory To assess results reliably it is wise to carry out the test on a normal subject once a week The results are recorded and any consistent trend in the normal value will be obvious Results on patients can then be compared with 20 most recent observations

(*b*) Method of Dale and Laidlaw (1911)

By this method the clotting time of capillary blood is measured

Small glass tubes 2 cm in length of wide capillary bore are prepared One end of each tube is partially closed by holding for a few seconds in a bunsen flame When the tube has cooled a small piece of lead shot is inserted into the tube and the other end partially closed to retain the lead shot The average weight of the lead shot should be about 0 006 gm The piece of lead shot should be freely mobile when the tube is tilted [1]

Capillary blood is obtained from a finger prick The first drop of blood to emerge is allowed to run into the tube The tube is held immersed in a water bath at 37° C using forceps the ends of which have been encased in rubber tubing to support the tube The tube is tilted until the lead shot remains stationary The clotting time is recorded from the time at which the blood enters the capillary tube to the time at which the lead shot ceases to move

By this method the clotting time of normal blood usually lies between 1½ and 3 minutes

(c) Method of Lee and White using silicone-coated tubes

The test is carried out in exactly the same manner as the Lee and White test using glass tubes with the exception that the tubes are treated with silicone The normal clotting time varies with the technique but is usually 18-25 minutes Minor coagulation defects may become much more obvious with this method

2 PROTHROMBIN CONSUMPTION TEST

(a) *Clotted Venous Blood*

The blood collected for the Lee and White coagulation time test is used When coagulation has occurred the blood in the four Lee and White clotting tubes is allowed to stand at 37° C for 50 minutes This time is measured from the completion of coagulation and not from the time at which blood is withdrawn from the patient The clot is then gently freed from the sides of the tubes with a wooden stick and the tubes are centrifuged at 1500 r p m for five minutes to obtain the serum The serum from the four tubes is collected with a Pasteur pipette and pooled in one tube At exactly one hour after coagulation has occurred a prothrombin consumption test is carried out.

Performance of Test

0 4 ml amounts of fibrinogen are placed in each of four tubes

[1] These tubes can be obtained from R. B Turner & Co 9-11 Eagle Street, Southampton Row London.

(measuring 2½ in × ⅜ in) and these are placed in a water bath at 37° C Another small tube of the same size is placed in the water bath and 0 2 ml of the pooled serum is placed in this tube 0 2 ml of 0 85 per cent NaCl and 0 2 ml of brain thromboplastin is added and as rapidly as possible thereafter 0 2 ml M/40 $CaCl_2$ is added starting a stop-watch at this instant The contents of the tube are thoroughly mixed and at exactly 30 seconds after the beginning of the test 0 1 ml of the mixture is removed with a graduated Pasteur pipette and rapidly blown out into one of the two tubes containing fibrinogen As this addition is made a second stop-watch is started and the coagulation time of the fibrinogen is recorded At 60 seconds a further 0 1 ml of the mixture is added to the second fibrinogen tube and the clotting time is recorded The test is carried out in exactly the same way using the patient s citrated plasma instead of serum In this test the mixture coagulates because the plasma contains fibrinogen The coagulum which forms after about 20 seconds must be removed with a wooden stick as rapidly as possible At exactly 30 and 60 seconds 0 1 ml of the defibrinated mixture is added to a tube containing fibrinogen and the clotting times of the fibrinogen recorded as before

Expression of Results

A method of expressing results can best be understood from an example Four clotting times are obtained The minimum clotting time for the plasma and serum are used to calculate the index In a normal blood minimum clotting times shown below were obtained From the clotting times of the fibrinogen a Prothrombin Consumption Index is calculated by dividing the shortest clotting time obtained with plasma by that with serum and multiplying the result by 100 In the example quoted the index would be $\frac{16}{160} \times 100 = 10$ per cent

TABLE 44

PROTHROMBIN CONSUMPTION TEST
TYPICAL RESULT IN A NORMAL INDIVIDUAL

Incubation time (secs.)	60
Plasma time (secs)	16
Serum time (secs)	160

Notes

In carrying out the prothrombin consumption test it is important that the blood should be collected with a syringe and needles of standard size and that exactly 1 ml of blood *should have been placed* in each of the four small tubes used for collection of blood Deviations from this procedure may have a surprisingly large effect on the results of the test For example if the blood is collected into one large container the results of the test may be much too high or, if a needle of small gauge is used the results of the test may be too low

Even with these precautions the results with normals are not constant and it is wise as with the *Lee and White clotting time* to carry out the test once a week on normal blood the normal range is thus always known with certainty The test has never been known to record more than 40 per cent in normal blood and in our laboratory none of the normal results for the last two years have exceeded 20 per cent Of 77 haemophilic patients seen by us all but 18 had an index above 50 per cent in many instances values above 100 per cent are found In a few patients the index may be below 20 per cent It should be noted that an abnormal result in this test will also be obtained in the blood of Christmas disease patients when the platelet count is below 50 000–100 000 per cu mm in functional platelet deficiency and in patients with inhibitory substances in their blood

The serum obtained after centrifuging the clotted blood at 50 minutes after clotting may be citrated by adding one part of 3 8 per cent sodium citrate solution to four parts of serum The conversion of prothrombin to thrombin is halted by the addition of citrate and the test can then be carried out at leisure

(b) Capillary Blood

Capillary blood is collected from finger prick or heel prick in an infant About 0 25–0 5 ml of whole blood is required This blood is collected into a tube measuring 2½ in × ⅜ in and allowed to clot in a water bath 37° C After incubation for 50 minutes the serum is separated as described before and at 1 hour after clotting the Prothrombin Consumption Test is carried out on the serum The test as carried out on the serum may be compared with that carried out on citrated plasma from the patient where that is possible or if plasma cannot be collected from the patient, plasma from a normal subject may be used. The results of this test are expressed in exactly the same way as the test carried out on venous blood.

Merskey (1950b) found that twenty-seven out of forty-two haemophilic patients gave an abnormal result i e Prothrombin Consumption Index of more than 40 per cent This test is not so reliable as the test used with venous blood but it is useful for the diagnosis of haemophilia in small infants

3 CALCIUM CLOTTING TIME

To 0 1 ml of citrated plasma is added 0 1 ml of 0 85 per cent saline The tube is placed in a water bath at 37° C and 0 1 ml of M/40 $CaCl_2$ is added A stop watch is started as the $CaCl_2$ is added and the time taken for coagulation to occur is recorded. With normal plasma the clotting time varies from 90 to 250 seconds

4 BLEEDING TIME (IVY S TECHNIQUE)

A sphygmomanometer cuff is placed around the patient s upper arm and the pressure is raised to 40 mm of mercury

With the Frank s spring lancet set at 3 mm three small punctures are made along the outer (extensor) surface of the patient s forearm taking care to avoid any scar tissue or superficial veins

With three filter paper discs one for each puncture-wound the three bleeding points are gently blotted without rubbing every 15 seconds This process is continued until all bleeding ceases

The average of the time taken for the bleeding to stop from the three puncture-wounds is taken as the patient s bleeding time A convenient method of estimating this is to add the total number of blood blots on the three filter papers and divide by twelve This gives the bleeding time in minutes (Normal = 2½ to 7 minutes)

5 TOURNIQUET TEST

A sphygmomanometer cuff is placed round the patient s upper arm and inflated to a pressure of between 70-90 mm of mercury This pressure is maintained for 5 minutes at the end of this time the pressure is released and the cuff removed The arm is inspected and the number of petechial haemorrhages is counted In normal people 0-10 petechiae may be found

6 PLATELET COUNT (METHOD OF BRECHER AND CRONKITE 1950)

1 per cent ammonium oxalate is required for this test It should be stored in the cold to discourage the growth of contaminants and filtered immediately before use

Method The patient s ear (or finger) is warmed by rubbing with

dry wool The ammonium oxalate is drawn up to the 0 5 mark of a white cell counting pipette The ear (or *finger*) *is pricked and a free* flow of blood obtained blood is then sucked up into the white cell pipette until the level of the oxalate previously introduced into the pipette reaches the 1 mark The volume of blood is thus 0 5 The pipette *is then filled to the 11 mark* with oxalate solution The pipette is shaken for 3 minutes and after discarding the first one or two drops a Neubauer counting chamber is filled The chamber is placed in a Petri dish containing damp filter paper and left for at least 20 minutes The platelets encountered in 80 small squares are counted using a 1/6th objective If N is the number of platelets counted then $N \times 100$ is the number per c mm of blood

Counting is possible under 1/6th objective with condenser lowered to make the platelets refractile Counting is very much more accurate if a phase contrast system is used In difficult cases with much particulate matter counting may be impossible without a phase contrast microscope

7 ONE-STAGE PROTHROMBIN TIME

0 1 ml of citrated normal plasma (Appendix III B 1) and 0 1 ml of extracted brain (Appendix III B11) are mixed in a tube measuring $2\frac{1}{2}$ in $\times$ $\frac{3}{8}$ in and placed in a water bath at 37 C 0 1 ml of *M/40* $CaCl_2$ is added and a stop watch is started The tube is tilted at frequent intervals until a firm clot appears when the stop watch is stopped and the time recorded The test is carried out in duplicate on normal and pathological plasma

With this method the normal plasma will clot in 11-20 seconds The variable clotting time of normal plasma is caused by the differing activity of different batches of brain extract with any one batch the clotting time is reasonably constant The comparison of abnormal and normal clotting times may be made either by the index or the ratio methods discussed in Chapter XVIII The use of dilution curves is not now recommended

8 PROTHROMBIN AND PROCONVERTIN METHOD (MODIFIED FROM OWREN AND AAS 1951)

Reagents

$BaSO_4$ treated Ox Blood

Ox blood is collected into 2 5 per cent potassium oxalate in the proportion of 1 part of oxalate to 9 parts of blood Plasma is obtained

by centrifuging and this is treated with $BaSO_4$ (Appendix III A10) 2 gm $BaSO_4$ being added for each 100 ml of plasma The mixture is stirred mechanically for 30 minutes at room temperature and the $BaSO_4$ removed by centrifuging A further adsorbtion is done with 2 gm $BaSO_4$ per 100 ml of plasma The $BaSO_4$ prepared as described in Appendix III is not so active as that of the Scandinavian workers and some prothrombin may remain unless two adsorbtions are done The finally centrifuged absorbed plasma is stored at −20 C in amounts required for 1 day s work Samples should not be used and refrozen

Brain Extract See Appendix III B12

Diluting Solutions

A	3 13 per cent trisodium citrate (dihydrate)	240 ml
	Distilled water	760 ml
B	Owren s buffer	200 ml
	Solution A	200 ml
	0 9 per cent saline	600 ml
C	3 13 per cent trisodium citrate	100 ml
	0 9 per cent saline	600 ml

Anticoagulant for collection of Blood

3 13 per cent trisodium citrate	250 ml
Heparin	25 mg (100 units per mg)
Merthiolate	25 mg

The heparin prevents alterations in clotting time which may occur in samples sent by post The blood is collected in proportion of 1 part of anticoagulant to 9 parts of blood and plasma is obtained by centrifuging

Normal Control Plasma

Blood is obtained from 10 normal subjects using the special anticoagulant the plasma is separated after centrifuging and pooled The pooled plasma is gently but thoroughly mixed and dispensed in 1 ml amounts into very clean glass tubes and stored at −20 C The tubes should be stoppered

Performance of Test

The plasma sample to be tested is diluted 1 in 10 using diluting fluid B 0 1 ml of diluted plasma is mixed with 0 1 ml of $BaSO_4$ treated ox plasma 0 1 ml of brain extract and 0 1 ml of $CaCl_2$ of

optimum concentration A stop watch is started as the $CaCl_2$ is added The normal clotting time by this method is 30-40 seconds as a rule 2 or even 4 tests may be carried out simultaneously if several stop watches are available

The calcium chloride concentration in this test must be optimal The optimum must be determined for each new batch of ox plasma or brain extract This is done by making a dilution curve (see below) with $CaCl_2$ concentrations M/10 M/20 and M/30 and selecting the concentration which gives minimum clotting times We usually find the M/20 to be optimum

Preparation of the Dilution Curve

Dilutions of the pooled normal control plasma are made 1 in 2 1 in 4 1 in 8 and 1 in 16 using diluting fluid C The undiluted plasma and these dilutions are then all diluted 1 in 10 using diluting fluid B to give final dilutions of 1 in 10 1 in 20 1 in 40 1 in 80 1 in 160 These two fluids are used to ensure a constant ionic strength for varying dilutions The test is carried out in duplicate for each of the dilutions The clotting times are then plotted against plasma concentration on double logarithmic graph paper when a straight line should be obtained (Fig 51)

The dilution curve should be repeated with every new reagent and each day two dilutions of normal plasma (e g 1/10 and 1/20) should be tested

Interpretation of Results

The clotting times are interpreted using the dilution curve prepared with normal plasma The method is used for the control of anticoagulant therapy with the dicoumarin group of drugs For this purpose the therapeutic range lies between 10 and 20 per cent of normal as read from the curve The method undoubtedly requires more attention to detail than the unmodified one-stage prothrombin time and it may be claimed that it is too complicated for routine laboratories In fact the actual test is no more difficult to perform than the one-stage prothrombin time The only serious complication is the preparation of prothrombin-free ox plasma

9 ONE-STAGE PROTHROMBIN TIME (MICRO-METHOD)

This method is included for use on patients from whom a venous sample cannot be obtained The method is not recommended for

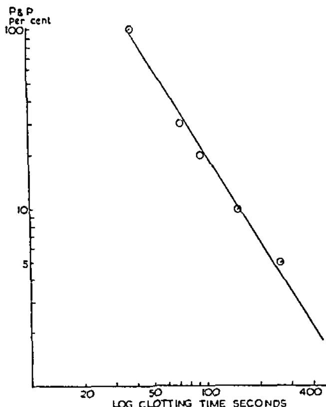


Fig 51 Dilution curve of normal pl sma as used in the prothrombin and proconvertin (p nd p) method of Owren a d Aas (1951) The log of the concentration of normal plasma t king the 1 in 10 dilution as 100 per cent is plotted ag inst the log of the clotting time in seconds

general use it is technically troublesome to obtain sufficient blood and the results are not very reliable

3 8 per cent trisodium citrate is drawn into a white cell counting pipette until it reaches the 1 mark A finger or ear previously warmed to obtain a good circulation is pricked deeply and free-flowing blood is drawn up to fill the pipette to the 11 mark The blood is then immediately blown out into a small tube and the pipette filled to the 11 mark with citrate saline (1 part of 3 8 per cent trisodium citrate and 5 parts of 0 85 per cent saline) The citrate saline is blown into the blood and the contents of the tube mixed

After centrifuging the plasma is tested by the one-stage method The test is repeated using normal blood when the patient s and normal clotting times can be compared by the ratio or index method (see Chapter XVIII)

10 QUALITATIVE TEST FOR FACTOR V DEFICIENCY

Normal citrated plasma is treated with $Al(OH)_3$ to remove prothrombin and Factor VII (Appendix III B 3) This plasma contains Factor V in excess but very little prothrombin or Factor VII 0 1 ml of $Al(OH)_3$ treated plasma is added to 0 9 ml of the patient s plasma and the mixture is tested by the one-stage prothrombin time If the clotting time of the mixture is considerably shorter than that of the patient s plasma alone then it is probable that the latter is deficient in Factor V

11 QUANTITATIVE TEST FOR FACTOR V DEFICIENCY

A quantitative test for Factor V deficiency requires a supply of Factor V deficient plasma if a simple test is to be devised Usually this is not available and either Factor V deficient plasma must be made artificially or an elaborate procedure involving the use of purified prothrombin must be resorted to Only the former method will be described

Factor V deficient plasma is made by collecting normal blood into oxalate (Appendix III B 1) The plasma is separated by centrifuging and stored in the cold at 4 C for 1-2 weeks Most of the Factor V will now have deteriorated A one-stage prothrombin time is carried out on the plasma and the clotting time should exceed 60 seconds Mixtures of this plasma and normal plasma are then made to contain 5 to 50 per cent of normal plasma Similar mixtures are made with the patient s plasma and the Factor V deficient plasma The two sets of figures can be compared

12 QUALITATIVE TEST FOR FACTOR VII DEFICIENCY

0 1 ml of normal serum is added to 0 9 ml of the patient s plasma and the one-stage prothrombin time of the mixture is tested If the clotting time of the mixture with serum is considerably shorter than that of the patient s plasma alone Factor VII deficiency should be suspected This can be confirmed by showing that the patient s plasma has less than the normal ability to shorten the one-stage prothrombin time of the plasma of patients under treatment with the dicoumarin drugs Mixtures are made of (*a*) 0 1 ml of normal,

and 0 9 ml of dicoumarin plasma and (*b*) 0 1 ml of the patient s plasma and 0 9 ml of dicoumarin plasma The one-stage prothrombin time test is carried out on the two mixtures

13 QUANTITATIVE MEASUREMENT OF FACTOR VII (OWREN AND AAS 1951)

The reagents required for this test are a saline brain extract as prepared for the p and p method (Appendix III B 12) Seitz filtered ox plasma (Appendix III B 8) and diluting fluids as for the p and p method (Appendix IV 8)

Samples to be tested are diluted 1/20 a 1 in 2 dilution being made with fluid C and a 1/10 with solution B (for diluting fluids see Appendix IV 8) The clotting mixture consists of

0 1 ml of diluted plasma
0 1 ml of brain extract
0 1 ml of Seitz filtered ox plasma
0 1 ml of $CaCl_2$ *(optimum concentration)*

The results of the test are assessed from a dilution curve prepared as described in the p and p method (Appendix IV 8) and the optimum concentrations of brain and CaCl are similarly determined The results of this test are more satisfactory if the Seitz filtered ox plasma is replaced by plasma from a Factor VII deficient patient but this is usually not possible

14 TWO-STAGE PROTHROMBIN TEST

A suspension of acetone dried brain and a fibrinogen solution are used in this test (Appendix III B 2 and 11) The suspension of brain is prepared as for the one-stage prothrombin time and diluted 1/5 1/10 1/20 with 0 85 per cent saline The dilutions are tested by the one-stage prothrombin time test using normal plasma and a dilution giving a clotting time of 25-30 seconds is selected The use of diluted brain slows the speed of thrombin formation and the test is technically simpler

0 4 ml of undiluted normal plasma is mixed with 0 4 ml of diluted brain emulsion in a tube measuring 2½ in × ⅜ in and placed at 37 C 0 4 ml of M/40 $CaCl_2$ is added A stop watch is started as the CaCl is added At minute intervals starting at 1 minute 0 1 ml is removed from this incubation mixture and added to 0 4 ml amounts of fibrinogen previously pipetted into small tubes and

After centrifuging the plasma is tested by the one-stage method The test is repeated using normal blood when the patient s and normal clotting times can be compared by the ratio or index method (see Chapter XVIII)

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Samples to be tested are diluted 1/20 a 1 in 2 dilution being made with fluid C and a 1/10 with solution B (for diluting fluids see Appendix IV 8) The clotting mixture consists of

0 1 ml of diluted plasma
0 1 ml of brain extract
0 1 ml of Seitz filtered ox plasma
0 1 ml of $CaCl_2$ (optimum concentration)

The results of the test are assessed from a dilution curve prepared as described in the p and p method (Appendix IV 8) and the optimum concentrations of brain and $CaCl_2$ are similarly determined The results of this test are more satisfactory if the Seitz filtered ox plasma is replaced by plasma from a Factor VII deficient patient but this is usually not possible

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A suspension of acetone dried brain and a fibrinogen solution are used in this test (Appendix III B 2 and 11) The suspension of brain is prepared as for the one-stage prothrombin time and diluted 1/5 1/10 1/20 with 0 85 per cent saline The dilutions are tested by the one-stage prothrombin time test using normal plasma and a dilution giving a clotting time of 25-30 seconds is selected. The use of diluted brain slows the speed of thrombin formation and the test is technically simpler

0 4 ml of undiluted normal plasma is mixed with 0.4 ml of diluted brain emulsion in a tube measuring 2½ in × ⅜ in and placed at 37° C 0 4 ml of M/40 CaCl is added A stop watch is started as the $CaCl_2$ is added At minute intervals starting at 1 minute 0 1 ml is removed from this incubation mixture and added to 0 4 ml amounts of fibrinogen previously pipetted into small tubes and

placed in the water bath The clotting times of the substrate fibrinogen are recorded

The test is continued until the clotting time of the substrate fibrinogen exceeds 3 minutes The thrombin units are then read from a thrombin-fibrinogen dilution curve (Appendix IV, 15) in terms of clotting time and a curve relating thrombin units to incubation time is drawn The area enclosed by the curve is computed either with a planimeter, by the more laborious method of counting the squares on the graph paper or by weighing the cut-out areas The procedure is carried out on normal and abnormal samples and the area obtained for the abnormal is expressed as a percentage of the normal

A technical difficulty results from clotting in the incubation mixture When this occurs the clot must be removed It has been found that the removal of the clot can be achieved quite easily by winding it on to a wooden swab stick the end of which has been split and the halves slightly separated

15 THROMBIN-FIBRINOGEN DILUTION CURVE

The standard preparation of thrombin topical (Parke Davis) contains about 1000 units of thrombin per ml of solution This solution is diluted to contain 20 15 10 8 5 4 3 2 1 and 0 5 units of thrombin per ml No attempt need be made to ensure that the thrombin units correspond with those of any standard preparation but the proportional dilutions must be carefully made If human thrombin is used the dilutions are arranged to include clotting times from 10 to 100 seconds Maws thrombin may also be used if appropriate dilutions are made A series of tubes containing 0 4 ml of fibrinogen are placed in a water bath at 37 C and to these 0 1 ml of the thrombin solutions are added and in each case the clotting time is recorded The results of one experiment are shown in Fig 1 From the figure it will be seen that if the clotting times are plotted against the reciprocal of the concentration of thrombin a straight line passing almost through the origin fits the points reasonably well For the thrombin-fibrinogen reaction it is possible to relate the clotting time to the concentration of thrombin as follows

$$C\,T = \frac{K}{T}$$

where C T = clotting time
and T = concentration of thrombin

From this curve clotting times of 0 4 ml amounts of fibrinogen can be read in terms of thrombin units These thrombin units have no absolute significance but are useful for comparing relative amounts of thrombin formed in particular experiments and in the two-stage prothrombin test

16 CLOT RETRACTION

Apparatus A graduated centrifuge tube reading to 0 1 ml and a glass rod about 5½ in in length with a series of expansions at about ½-in intervals and with a perforated cork fixed at one end The glass rod may be replaced by a spiral made of wire which has a diameter of 1-1 5 mm The test is easier to carry out if a siliconed tube is used

Technique Rather more than 5 ml of blood is withdrawn into a dry syringe by means of a clean venepuncture It is placed straight away into a clean test tube without frothing and 5 ml delivered by pipette into the graduated centrifuge tube The glass rod or wire spiral is then placed in the tube and held in a vertical position by means of the cork. With the wire spiral a cork is usually unnecessary

The centrifuge tube is then incubated at 37 C preferably in a water bath, and examined from time to time until the blood has clotted firmly It is then left undisturbed at 37 C for a further hour at the end of which time the rod or wire around which the clot should have retracted is carefully lifted in the tube and allowed to drain for a minute or so and then removed The volume of expressed serum and cells is read off directly from the graduations on the tube The volume expressed as a percentage of 5 ml represents the percentage retraction of the clot

The centrifuge tube must be absolutely clean otherwise the clot may adhere to the sides This adherence is less troublesome if siliconed tubes are used If there is partial adherence the clot may be gently freed with a platinum loop and reincubated for a further hour without introducing much error

In anaemia retraction is generally increased due to the smaller red-cell volume the relation between the two variables being approximately linear The blood platelet level below which impaired retraction may be expected is said to be about 100 000 cu mm Other conditions in which there may be impaired retraction include jaundice pneumonia myelomatosis and polycythaemia

In 50 subjects (27 men and 23 women) retraction varied from 48 to 64 per cent of serum expressed with a mean of 54 7

placed in the water bath The clotting times of the substrate fibrinogen are recorded

The test is continued until the clotting time of the substrate fibrinogen exceeds 3 minutes The thrombin units are then read from a thrombin-fibrinogen dilution curve (Appendix IV 15) in terms of clotting time and a curve relating thrombin units to incubation time is drawn The area enclosed by the curve is computed either with a planimeter by the more laborious method of counting the squares on the graph paper or by weighing the cut-out areas The procedure is carried out on normal and abnormal samples and the area obtained for the abnormal is expressed as a percentage of the normal

A technical difficulty results from clotting in the incubation mixture When this occurs the clot must be removed It has been found that the removal of the clot can be achieved quite easily by winding it on to a wooden swab stick, the end of which has been split and the halves slightly separated

15 THROMBIN-FIBRINOGEN DILUTION CURVE

The standard preparation of thrombin topical (Parke Davis) contains about 1000 units of thrombin per ml of solution This solution is diluted to contain 20 15 10 8 5 4 3 2 1 and 0 5 units of thrombin per ml No attempt need be made to ensure that the thrombin units correspond with those of any standard preparation but the proportional dilutions must be carefully made If human thrombin is used the dilutions are arranged to include clotting times from 10 to 100 seconds Maws thrombin may also be used if appropriate dilutions are made A series of tubes containing 0 4 ml of fibrinogen are placed in a water bath at 37 C and to these 0 1 ml of the thrombin solutions are added and in each case the clotting time is recorded The results of one experiment are shown in Fig 1 From the figure it will be seen that if the clotting times are plotted against the reciprocal of the concentration of thrombin a straight line passing almost through the origin fits the points reasonably well For the thrombin-fibrinogen reaction it is possible to relate the clotting time to the concentration of thrombin as follows

$$C\,T = \frac{K}{T}$$

where C T = clotting time
and T = concentration of thrombin

the two tubes are allowed to stand at room temperature for 10 min Both tubes are centrifuged at 2000 r p m for 10 min and the volumes of precipitate are compared

If the normal specimen produces 0 1 ml of precipitate and the patient s blood 0 01 ml then the patient would be said to have 10 per cent of the normal amount of fibrinogen

It is unwise to rely entirely on this test for the diagnosis of obstetric defibrination The Lee and White clotting time test (Appendix IV 1) the one-stage prothrombin time test (Appendix IV 7) and the reaction of plasma to thrombin (Appendix IV 22) should also be carried out The clot in the Lee and White test is either absent or markedly lacking in solidity the clot in the one-stage test is either absent or a fine wisp of fibrin which rapidly contracts to an almost invisible white pinhead-sized clot the patient s plasma usually will not clot on the addition of strong thrombin solutions

18 DEMONSTRATION OF FIBRINOLYSIS IN PLASMA SAMPLES

Reagents

(*a*) Buffered Saline (See Appendix III A 8)

(*b*) M/20 $CaCl_2$

(*c*) Thrombin Solution containing approximately 20 units per ml using a sample of thrombin topical of Parke Davis as standard

Technique

Performance of Test

0 4 ml of citrated plasma (Appendix III B 1) is added to 6 ml of buffered saline to give a dilution 1/16 Two sets of three tubes are prepared as follows

(*a*) 1 6 ml diluted plasma

(*b*) 0 8 ml diluted plasma + 0 8 ml buffered saline

(*c*) 0 4 ml diluted plasma + 1 2 ml buffered saline

To each tube of one set is added one drop of thrombin and to each of the second set 0 1 ml of M/20 $CaCl_2$ The contents of the tubes are mixed by inverting

The six tubes are placed in a water bath at 37 C together with a similar set of tubes prepared from normal plasma. The test should be made at a standard time after withdrawing the blood because the fibrinolytic enzyme may disappear from the plasma after storage The tubes are observed to ensure that coagulation occurs and then

17 MEASUREMENT OF FIBRINOGEN

(a) *Biochemical Method*

Venous blood is collected into a bottle containing dried sodium or potassium oxalate as anticoagulant The plasma is obtained by centrifuging

Performance of Test

1 ml of plasma is mixed with 25 ml of 0 85 per cent saline and 1 ml of 2 5 per cent $CaCl_2$ solution is added After 1 hour at room temperature the fibrin clot is filtered off and washed three times with 0 85 per cent saline The clot is then transferred to a digestion flask and digested with 1 ml of conc H_2SO_4 The digestion is continued over a moderate flame for four hours If the digest is not then perfectly clear 2 drops of H_2O_2 (100 vols) are added carefully and the digestion continued for another hour

The digest is transferred to a micro Kjeldahl distillation apparatus and the flask washed with 3-4 changes of distilled water which are added to the digest in the apparatus The ammonia is driven off by the action of an excess of 40 per cent NaOH and collected in a flask containing 10 ml of N/50 H_2SO_4 The excess of H_2SO_4 is titrated with N/50 NaOH using methyl red as indicator (10 − titration figure) $\times$ 100 $\times$ 0 28 = mg of fibrinogen N per 100 ml

Quick (1951a) has given an alternative method in which the fibrin is dissolved by boiling with NaOH and the amount of colour developed with Folin-Ciocalteu s phenol reagent is determined

(b) *Rapid method for the Demonstration of Fibrinogen deficiency* (for use in patients with acute defibrination such as occurs in some obstetric cases)

A half-saturated solution of ammonium sulphate is made by dissolving 26 gm of $(NH_4)_2SO$ in 50 ml of distilled water and making the volume up to 100 ml with distilled water It is better to make the solution in this way than to use a 1 in 2 dilution of a supposedly saturated solution of ammonium sulphate

Citrated plasma is obtained from the patient and from a similar patient in whom there is no evidence of any bleeding tendency (Appendix III B 1)

2 ml of the patient s plasma is placed in a modified Esbach s centrifuge tube (Hopkins type of Vaccine tube Gallenkamp) and 2 ml of half-saturated ammonium sulphate is added and the contents of the tube is mixed 2 ml of the control plasma is similarly treated and

mixture is cooled to room temperature and Na_2CO_3 solution (12 5 per cent w/v) added to the 8 0 ml mark (If it is inconvenient to proceed at once with the development of the colour the tubes may be left at this stage – if necessary overnight) Copper sulphate solution (1 0 ml) is added and the mixture stirred with the glass rod The diluted Folin-Ciocalteu s reagent (1 0 ml) is then added at a steady rate with stirring (we find a 1 0 ml all-glass syringe useful for this addition) After standing for at least ½ hour the colour is read in a photo-electric colorimeter using a red filter against a blank prepared using 2 0 ml 0 1 N NaOH and the rest of the reagents as described The amount of colour is described in terms of the amount of pure tyrosine required to produce the same deflection of the galvanometer when treated with the same reagents (It is well recognized that the colour produced by the dissolved clot is due to other compounds in addition to tyrosine) The amount of colour is proportional to the amount of clot and lysis may be followed by plotting a graph connecting the amount of clot (expressed as μg Cu-Tyrosine) with the time of incubation.

20 MEASUREMENT OF ANTITHROMBIN (MODIFICATION OF THE METHOD OF ASTRUP AND DARLING 1942)

A 1/5 or 1/10 dilution of citrated plasma (Appendix III B 1) is made Mixtures of the diluted plasma and saline are made as follows

Diluted plasma	ml	0	0 05	0 1	0 15	0 1	0 3
Saline	ml	0 3	0 25	0 2	0 15	0 3	0

To each of these mixtures is added 1 ml of a thrombin preparation The mixtures are incubated at 37 C for 15 minutes and then the tubes are placed in water at the temperature of melting ice to stop the neutralization of thrombin 0 1 ml amounts of each of the mixtures is then added to 1 ml. amounts of fibrinogen in tubes kept in a water bath at 37 C The clotting times of the fibrinogen are recorded The concentration of thrombin is adjusted so that when a sample is transferred from the control mixture (containing no plasma) to the fibrinogen the fibrinogen clotting time lies between 10 and 15 seconds The concentration of plasma is adjusted so that the fibrinogen clotting time with the highest concentration of plasma does not exceed 60 seconds

It has been found that the concentration of antithrombin may be expressed by the formula

left for twenty-four hours for lysis of the clot (During this time the tubes may be observed at intervals and the lysis time recorded the reciprocal of the lysis time gives an approximate measure of fibrinolytic activity)

19 MEASUREMENT OF FIBRINOLYTIC ACTIVITY (BIDWELL 1953)

Reagents

Buffered saline (see Appendix III A 8)

Thrombin purified by acetone precipitation as described in Appendix III B 6 The freeze dried precipitate is dissolved in 0 85 per cent saline to give approximately 20 units per ml

0 1 N NaOH filtered before use

12 5 per cent w/v Na_2CO_3 filtered before use

0 01 M $CuSO_4$

Folin-Ciocalteu s phenol reagent (B D H) diluted with two volumes of water before use

Technique

Performance of Test

A series of tubes is prepared each containing 4 8 ml buffered saline To the first tube add 0 2 ml citrated plasma (Appendix III, B 1) and mix with a glass rod of which the lower end is roughened along at least 1 in of its length The rod is left in the mixture which is placed in a water bath at 37 C After exactly 5 minutes thrombin (0 2 ml) is added and mixed rapidly with the stirring rod which is left in situ The other tubes in the series are prepared in the same way and the clots are incubated at 37 C for various times e g ½ 1 1½ hours the incubation time is reckoned from the time of addition of the thrombin

Meanwhile a corresponding number of tubes graduated at 8 0 ml and each containing approximately 10 ml 0 85 per cent saline are placed in a bath of melting ice When the clot has been incubated for the chosen time it is collected by gently winding it on to the glass rod and transferred to a tube of ice cold saline The clot is washed with two more lots of approximately 10 ml ice cold saline (i e about 30 ml altogether) allowing 15-30 minutes standing in the ice-water bath between each washing The last saline washings are poured away and 2 0 ml 0 1 N NaOH added down the rod The tube is then immersed in a boiling water bath for 1-2 minutes taking care that the warm NaOH comes into contact with all the clot The

The values for t_o/t are plotted against the concentration of plasma and an approximate straight line is drawn through the points (Fig 52) A corrected value for t /t is then obtained from this line Thus when 0 02 ml plasma (0 1 ml of a 1/5 dilution) is used t_o/t_a would be read as 0 76 instead of 0 82

The concentration of antithrombin is then calculated

$$c = \frac{30}{0\ 02}(1 - 0\ 76) = 360 \text{ units}$$

This is obviously a complicated technique but it is doubtful whether simpler methods are reliable

21 MEASUREMENT OF HEPARIN (JAQUES AND CHARLES 1941)

Tubes with uniform bore of about 8 mm are marked at the level to contain 1 ml A standard heparin solution containing 0.4 units per ml is prepared Oxalated beef blood is used for the test and is collected in 1-litre flasks containing 10 ml of 20 per cent neutral potassium oxalate Thrombin prepared by Mellanby s method (Chapter II) was used in the original test but any preparation of thrombin e g thrombin topical Parke Davis may be used

In a preliminary test the most suitable concentration of thrombin is selected 0 5 ml of oxalated blood is placed in a number of tubes 0 25 ml of heparin solution is added to each 0 15 ml of saline is added to each and 0 1 ml of different concentrations of thrombin are added The concentration of thrombin which is just sufficient to clot the blood in 15 minutes is selected

In the test 0 2 0 22 0 25 0 27 and 0 32 ml of heparin solution are placed in tubes The volume is made up to 0 4 ml with saline and to each tube is added 0 5 ml of oxalated blood To a second series of tubes mixtures containing dilutions of the unknown heparin preparation are added. The approximate heparin concentration in the unknown is first determined by making widely differing dilutions and in a second experiment dilutions comparable to those of the standard can be prepared

The tubes are placed in a water bath at 25 C for 10 minutes and then 0 1 ml of the selected thrombin preparation is added The contents of the tubes are mixed and the tubes allowed to stand at 25 C for 15 minutes The result is then read The end point is taken as the tube in which coagulation just fails to occur Usually it is found that one tube is solidly clotted the next contains a friable

$$c = \frac{a}{n}\left(1 - \frac{t_0}{t_n}\right)$$

where c is the concentration of antithrombin

a is the concentration of thrombin

n is the amount of plasma

t_0 is the fibrinogen clotting time with no added plasma

t_n are the clotting times of the fibrinogen with various concentrations of added plasma

The results of one test are shown in Table 45

TABLE 45

MEASUREMENT OF ANTITHROMBIN

Saline ml	0 3	0 2	0 15	0 1	0
Plasma diluted 1/5 ml	0	0 1	0 15	0 2	0 3
Thrombin (30 units/ml) ml	1	1	1	1	1
Clotting time of fibrinogen (seconds)	15	18 3	25 3	28 7	50
t_0/t_n	1 0	0 82	0 59	0 52	0 3

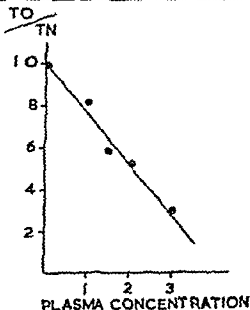


Fig 52 Measurement of anti-thrombin

A mixture is made to contain 0 2 ml of $Al(OH)_3$ treated normal plasma diluted 1 in 5 with 0 85 per cent saline 0 2 ml of normal serum diluted 1 in 10 with 0 85 per cent saline 0 2 ml of platelet suspension and in addition 0 2 ml of 0 85 per cent saline then 0 2 ml of M/40 $CaCl_2$ The thromboplastin generation test is carried out on this mixture using normal plasma as substrate The test is repeated replacing the additional 0 2 ml of 0 85 per cent saline by 0 2 ml of various saline dilutions of the patient s $Al(OH)_3$ treated plasma ranging from 1 in 5 to 1 in 100 according to the potency of the anticoagulant By recording the dilution at which no inhibitory effect is recorded a rough estimate of the anticoagulant activity can be made

TABLE 46

DEMONSTRATION OF ANTICOAGULANT

		Proportions of Mixtures				
Normal plasma		1	0 9	0 5	0 1	0
Patient s plasma		0	0 1	0 5	0 9	1
Clotting time (seconds)	Immediately	160	240	224	300	570
	after 2 hrs	—	440	630	555	—

With either of these tests it may occasionally be necessary to incubate the normal and abnormal reagents together for some time (15 min to 2 hours) before testing to demonstrate the anticoagulant the action of the anticoagulant taking some time to be effective Using the thromboplastin generation test it may be necessary to use the patient s $Al(OH)_3$ treated plasma undiluted because the effect of some inhibitors may be much reduced by dilution 1 in 5

25 DEMONSTRATION OF AN INHIBITOR OF ANTIHAEMOPHILIC GLOBULIN

Occasionally a patient with a haemorrhagic diathesis may have a prolonged whole blood clotting time because the blood contains an inhibitor which inactivates the antihaemophilic globulin The inhibitor may sometimes be identified by the following test

The patient s and normal plasma are mixed in equal parts and the mixture incubated at 37° C for 2 hours At the end of this time the mixture is diluted 1/10 1/100 1/1000 with 0 85 per cent saline

incomplete clot and in the next coagulation is absent By comparison with the standard the amount of heparin in the unknown sample can be calculated

22 *THROMBIN-FIBRINOGEN REACTION OF PLASMA*

A number of dilutions of a thrombin preparation are made 0 1 ml of each of the dilutions is added to 0 4 ml of citrated plasma (Appendix III B 1) and the clotting time recorded The test is carried out with normal and pathological plasma and the clotting times are compared There is no quantitative method for assessing the degree of abnormality

23 THE EFFECT OF TOLUIDINE BLUE ON THE THROMBIN-FIBRINOGEN REACTION

If the test of the thrombin-fibrinogen reaction has been found to be abnormal the abnormality may be due to the presence of heparin Heparin is neutralized by toluidine blue

A series of 0 1 ml samples of plasma are mixed with 0 1 ml of 0 85 per cent saline or 0 1 ml of toluidine blue solution containing 25 mg or 50 mg per cent toluidine blue 0 1 ml of different thrombin dilutions are then added to each of the mixtures and the clotting times recorded The test is carried out on the normal and abnormal plasma samples and the results compared If there is a close approximation of the results with normal and abnormal plasma when toluidine blue is added then it is likely that heparin is present

24 DEMONSTRATION OF INHIBITORS OF PLASMA THROMBOPLASTIN

(*a*) *Using the Calcium Clotting Time Test* (Appendix IV 3)

The presence of an inhibitor may be demonstrated by testing mixtures of normal and the patient s plasma by the calcium clotting time method Mixtures are prepared as follows

Normal plasma	0	0 1	0 5	0 9	1
Patient s plasma	1	0 9	0 5	0 1	0

These mixtures are tested by the calcium clotting time method immediately and after two hours incubation at 37 C The results of an experiment carried out in this way are shown in Table 46

(*b*) *Using the Thromboplastin Generation Test* (Appendix IV 28)

$Al(OH)_3$ treated plasma is prepared from both normal and the patient s plasma The other reagents are prepared from normal blood

A mixture is made to contain 0 2 ml of $Al(OH)_3$ treated normal plasma diluted 1 in 5 with 0 85 per cent saline 0 2 ml of normal serum diluted 1 in 10 with 0 85 per cent saline 0 2 ml of platelet suspension and in addition 0 2 ml of 0 85 per cent saline then 0 2 ml of M/40 $CaCl_2$ The thromboplastin generation test is carried out on this mixture using normal plasma as substrate The test is repeated replacing the additional 0 2 ml of 0 85 per cent saline by 0 2 ml of various saline dilutions of the patient s $Al(OH)_3$ treated plasma ranging from 1 in 5 to 1 in 100 according to the potency of the anticoagulant By recording the dilution at which no inhibitory effect is recorded a rough estimate of the anticoagulant activity can be made

TABLE 46

DEMONSTRATION OF ANTICOAGULANT

		Proportions of Mixtures				
Normal plasma		1	0 9	0 5	0 1	0
Patient s plasma		0	0 1	0 5	0 9	1
Clotting time (seconds)	Immediately	160	240	224	300	570
	after 2 hrs.	—	440	630	555	—

With either of these tests it may occasionally be necessary to incubate the normal and abnormal reagents together for some time (15 min to 2 hours) before testing to demonstrate the anticoagulant the action of the anticoagulant taking some time to be effective Using the thromboplastin generation test it may be necessary to use the patient s Al(OH) treated plasma undiluted because the effect of some inhibitors may be much reduced by dilution 1 in 5

25 DEMONSTRATION OF AN INHIBITOR OF ANTIHAEMOPHILIC GLOBULIN

Occasionally a patient with a haemorrhagic diathesis may have a prolonged whole blood clotting time because the blood contains an inhibitor which inactivates the antihaemophilic globulin The inhibitor may sometimes be identified by the following test

The patient s and normal plasma are mixed in equal parts and the mixture incubated at 37 C for 2 hours At the end of this time the mixture is diluted 1/10 1/100 1/1000 with 0 85 per cent saline

Samples of the patient's and normal plasma are similarly diluted A sample of haemophilic plasma is obtained and mixtures are made as follows

TABLE 47

Haemophilic plasma	*Patient*			*Equal parts of Patient/Normal*			*Normal*		
	1/10	1/100	1/1000	1/10	1/100	1/1000	1/10	1/100	1/1000
0 1	0 1								
0 1		0 1							
0 1			0 1						
0 1				0 1					
0 1					0 1				
0 1						0 1			
0 1							0 1		
0 1								0 1	
0 1									0 1

To each mixture is added 0 1 ml of M/40 $CaCl_2$ When low proportions of normal plasma are added to haemophilic plasma the calcium clotting time is shortened If the inhibitor has destroyed the antihaemophilic globulin then the dilutions of the patient s plasma and dilutions of the mixture of normal and patient s plasma should fail to shorten the clotting time of the haemophilic plasma This test depends on the assumption that the direct effect of the inhibitor is removed by dilution whereas the antihaemophilic globulin is active in high dilution

26 DEMONSTRATION OF AN INHIBITOR OF BRAIN THROMBOPLASTIN

(*a*) *Inhibition of the reaction between Brain Extract and Normal Plasma*

The one-stage prothrombin time test is carried out on a normal plasma sample on the patient s plasma and on a mixture of normal and abnormal plasma in equal parts Acetone dried brain (Appendix III 11) is used in this test If the clotting time of the mixture is more than 2-3 seconds longer than that of the normal plasma then an inhibitor is probably present The inhibitor may be heparin and this

possibility can be tested by adding solutions of toluidine blue to the abnormal plasma and repeating the one-stage prothrombin time If the presence of toluidine blue shortens the clotting time then heparin is probably present (see Effect of Toluidine blue on the thrombin-fibrinogen reaction Appendix IV 23)

(*b*) *Neutralization of Brain Thromboplastin* (Appendix III 11)

10–12 mixtures of 0 1 ml of plasma and 0 1 ml of brain thromboplastin suspension are prepared and placed in a water bath at 37 C At intervals one of the mixtures is recalcified and the clotting time recorded The test is carried out with normal and pathological plasma If the clotting times of the mixtures with the pathological plasma lengthen on incubation more markedly than those with normal plasma an abnormal inhibitor may be present

27 THROMBIN GENERATION TEST (MACFARLANE AND BIGGS 1953)

(*a*) *Using Whole Blood*

2 ml of venous blood is placed in a centrifuge tube containing 0 2 ml of 0 85 per cent saline the contents are mixed and the tube placed in a water bath at 37 C At intervals of 1 minute samples of 0 1 ml are withdrawn from this incubation mixture and added to 0 4 ml amounts of fibrinogen previously placed in the water bath The clotting times of the fibrinogen are recorded Using a thrombin-fibrinogen dilution curve the clotting times are interpreted in terms of thrombin units as described for the two-stage test (Appendix IV 15)

During the process of clotting the clot must be removed with a swab stick expressing as much serum from the clot as possible during the process

In experimental procedures the 0 2 ml amounts of 0 85 per cent saline may be replaced by other reagents (thrombin serum plasma etc) as required

(*b*) *Using Plasma* (Pitney and Dacie 1953)

For comparative experiments samples should be tested immediately they have been collected and normal and abnormal blood samples should be centrifuged together at about 1500 r p m for 5 minutes to avoid differences in platelet levels in the samples For she test 0 4 ml of plasma is mixed with 0 4 ml of 0 85 per cent taline and 0 4 ml of M/40 $CaCl_2$ is added The clot which forms in the incubation mixture can easily be removed on a wooden applicator

stick and the formation of thrombin is recorded as in the test for whole blood

28 THROMBOPLASTIN GENERATION TEST (BIGGS AND DOUGLAS 1953b) The reagents required for this test are

(a) $Al(OH)_3$ treated plasma This is made by adding 0 1 ml of $Al(OH)_3$ (Appendix III A 9) to 1 ml of plasma the mixture is allowed to stand at 37° C for 1 minute and the $Al(OH)_3$ removed by centrifuging For use the adsorbed plasma is diluted 1 in 5 with 0 85 per cent saline

(b) Platelet suspension – (See Appendix III B 10) The platelet suspension may be replaced by a chloroform extract of brain (Appendix III B 14) The optimum concentration of this extract must be determined

(c) Serum – (See Appendix III B 1) The serum is diluted 1 in 10 with 0 85 per cent saline for use It has been found that the activity of diluted serum tends to improve on standing The serum dilutions should be made up and allowed to stand at room temperature for at *least an hour before testing*

Performance of Test

0 3 ml of platelet suspension (or lipoid) are mixed with 0 3 ml of diluted $Al(OH)_3$ treated plasma 0 3 ml of diluted serum and 0 3 ml of M/40 $CaCl_2$ A stop-watch is started on the addition of $CaCl_2$ The tubes are incubated at 37 C and at one-minute intervals 0 1 ml amounts are removed from this incubation mixture and added together with 0 1 ml of M/40 $CaCl_2$ to 0 1 ml amounts of citrated plasma (Appendix III B 1) previously pipetted out and placed in the water bath *If graduated pasteur pipettes are held one in each hand* this simultaneous addition is not difficult The clotting times of the plasma samples are recorded It will be found that the samples transferred from the incubation mixture have little ability to clot fibrinogen the coagulant activity for plasma must therefore be attributed to the formation of thromboplastin It is found that minimum clotting times of 7-12 seconds are obtained in 3-5 minutes if normal reagents are used The amount of thromboplastin formed can be assessed from a thromboplastin dilution curve prepared as described in Appendix IV 29 The incubation mixture usually clots this clot which can easily be removed is due to the presence of traces of thrombin in the incubation mixture the amounts are too small to affect the substrate clotting times

29 PLASMA THROMBOPLASTIN DILUTION CURVE

Plasma thromboplastin is made as in the last section When a minimum substrate clotting time has been reached the mixture forming thromboplastin is placed at the temperature of melting ice Dilutions of this preparation are made and tested with the substrate as described in the last section A curve relating clotting time and concentration of thromboplastin is then drawn (see Fig 22)

30 THE MEASUREMENT OF ANTIHAEMOPHILIC GLOBULIN

(a) *Using the Calcium Clotting Time Test* (Appendix IV 3)

The antihaemophilic activity of a plasma or fibrinogen sample may be tested by observing the ability of the sample to shorten the calcium clotting time of haemophilic plasma

Dilutions of the test samples 1/10 1/100 and 1/1000 made with 0 85 per cent saline are prepared 0 1 ml of haemophilic plasma is pipetted into a number of tubes To the first is added 0 1 ml of 0 85 per cent saline To the subsequent tubes are added 0 1 ml of the dilutions of the samples to be tested To each of the tubes 0 1 ml of M/40 $CaCl_2$ is added and the clotting times of the mixtures are recorded. The clotting times with dilutions of a test sample may be compared with those obtained with dilutions of normal plasma

This test is simple but has disadvantages Firstly the ability of the test sample to shorten the in vitro clotting time of haemophilic plasma is no guide to the probable in vivo activity of the substance tested It is usually found that the 1/10 dilution of normal plasma shortens the clotting time of haemophilic plasma to normal whereas as much as 20 pints of transfused whole blood may be required to stop a haemorrhage in vivo

(b) *Using the Prothrombin Consumption Test* (Appendix IV 2)

A patient whose blood is to be tested for antihaemophilic activity and a haemophilic patient may be vene-punctured simultaneously and the blood of the two patients mixed in various proportions and the mixtures allowed to clot One hour after clotting the prothrombin consumption test may be carried out on the samples of serum The results may be compared with those obtained by mixing normal and haemophilic blood

This test has the disadvantage that it is technically difficult to achieve accurate measurement of the volumes of the two samples mixed

(*c*) *Using the Thrombin Generation Test* (Appendix IV, 27)

In the blood of haemophilic patients whose Lee and White clotting time exceeds 30 minutes no measurable thrombin is generated in the thrombin generation test When various proportions of normal plasma are added to the blood thrombin is generated but the time at which thrombin generation starts is delayed A rough quantitative measure of the antihaemophilic effect of a preparation may be obtained by comparing the effects of a test preparation with those of normal plasma

(*d*) *Using the Thromboplastin Generation Test* (Appendix IV, 28)

Thromboplastin is generated in a mixture of normal $Al(OH)_3$ treated plasma normal serum platelets and $CaCl_2$ When the normal $Al(OH)_3$ treated plasma is replaced by $Al(OH)_3$ treated haemophilic plasma thromboplastin formation is much reduced If the plasma of a severely affected haemophilic patient is available an assay method may be devised

TABLE 48

THROMBOPLASTIN GENERATION TEST USED FOR THE ASSAY OF ANTIHAEMOPHILIC GLOBULIN

Source of $Al(OH)_3$ Plasma	*Incubation time minutes*				
	1	2	3	4	6
	Clotting time seconds				
Normal	34	10	9	9	10
Severely affected Haemophiliac	39	35	32	30	34
Mildly affected Haemophiliac	36	19	15	15	14
10 per cent Normal in severely affected Haemophiliac	41	26	13	13	15
20 per cent Normal in severely affected Haemophiliac	39	20	12	11	12

The normal the severely affected haemophilic and the patient s $Al(OH)_3$ treated plasma samples are all diluted 1 in 5 with 0 85 per cent saline The normal and the severely affected haemophilic samples are used mixed in various proportions

Haemophilic	0	4	9	19	39	1
Normal	1	1	1	1	1	0

In this way samples containing 100 20 10 5 2 5 and 0 per cent of normal $Al(OH)_3$ treated plasma respectively are obtained These samples are used as $Al(OH)_3$ treated plasma in the thromboplastin generation test and the results provide a series of figures representative of different levels of antihaemophilic globulin The unknown sample is then tested and the results compared with the series containing known amounts of A H G With a little experience it is usually not necessary to test all of the mixtures it being obvious from preliminary tests that the result is above or below 10 per cent An example of the use of this method is given in Table 48

From this table the patient would be said to have slightly less than 10 per cent of antihaemophilic globulin

(*e*) *Using the Assay of Antihaemophilic Globulin Activity* (Biggs Eveling and Richards 1955)

The first two methods described are not very satisfactory The calcium clotting time test and the prothrombin consumption test are unduly affected by trace amounts of A H G All require haemophilic blood which is not always available

The assay procedure which gives a quantitative assay of antihaemophilic globulin is a modification of the thromboplastin generation test In principle a number of dilutions of normal and the test plasma are tested after incubation for a standard time as in the thromboplastin generation test

Reagents

Glyoxaline Buffer (see Appendix III A 6)

Factor V (see Appendix III B 5 The method using BaSO for adsorbtion gives the best activity)

Factor V is prepared from 250 ml of human oxalated plasma The material is dried by the lyophil drying method For testing 40-50 mg are dissolved in 8 ml of glyoxaline buffer at pH 7 3 If the Factor V is dissolved in saline the solution has a pH of 5 and is very unstable A 1/20 dilution of $Al(OH)_3$ adsorbed haemophilic plasma may be used if this is more convenient The plasma may be stored frozen solid at −20 C and diluted when required.

Normal Serum

Normal serum is used as a source of Factor VII and the Christmas factor Blood is allowed to clot at 37 C under sterile conditions The clotted blood is incubated at 37° C for a further 5-7 hours and

stored overnight at 4 C The serum is then removed and dried by the lyophil method For use 200 mg of the dried material are dissolved in 10 ml of glyoxaline buffer at pH 7 3 If the solution is made in saline the pH is found to be 9 5 The solution of serum tends to improve in activity on storage the solution was therefore routinely made up the evening before it was required and allowed to stand in the refrigerator overnight to allow maximum activity to develop A sufficiently stable activity is usually reached in about 4 hours

$CaCl_2$

Aqueous solutions M/40 and M/20 are used

Citrate Saline

One part of 3 8 per cent trisodium citrate and five parts of 0 85 per cent saline

Phospholipid (see Appendix III B 13)

Citrated Plasma (see Appendix III B 1)

Performance of Test

When the thromboplastin generation test is carried out using increasing dilutions of normal $Al(OH)_3$ *treated citrated plasma* as a source of A H G it is found that the minimum clotting time reached after incubation increases with the higher dilutions (see Table 49) It is believed that this minimum clotting time can be related to A H G content With any particular set of reagents the time at which a minimum is reached varies and the actual clotting times also vary With relatively concentrated normal plasma ($\frac{1}{2}$ to $\frac{1}{8}$ dilution) a constant minimum clotting time of 8–12 seconds is found With higher dilutions a progressive lengthening of the clotting time occurs and the test must be done using these higher dilutions Using normal plasma as a standard it is desirable to obtain a range of minimum clotting times between 12 to 15 seconds and 25 to 30 seconds When carrying out the test it is reasonably economical of time to test 4 dilutions of plasma at two incubation times A preliminary test must be carried out to determine the best dilutions of normal plasma to use and the best incubation times

For this test dilutions of normal $Al(OH)_3$ treated plasma from 1/16 to 1/128 *are made using citrate saline solutions as diluent*

0 1 ml of the 1/16 dilution is mixed with 0 1 ml of Factor V 0 1 ml of phospholipid 0 1 of serum and 0 1 ml of M/20 $CaCl_2$

TABLE 49

The preliminary test of the assay method to determine the time at which a minimum clotting time was reached

Dilution of Plasma	Incubation time in minutes			
	10	15	20	25
	Clotting time in seconds			
Normal Plasma 1/8	11	10	11	12
1/16	17	14	15	15
1/32	30	17	16	18
1/64	83	26	20	22
1/128	110	55	25	25
1/256	100	55	29	30
Haemophilic Plasma 1/2	27	24	25	25
1/4	44	30	29	30
1/8	60	37	35	35

As the $CaCl_2$ is added a stop watch is started This stop watch referred to as the incubation stop watch is used to time all the dilutions as described below When the mixture has incubated at 37 C for 10 minutes 0 1 ml is removed from the mixture and added together with 0 1 ml of M/40 CaCl to 0 1 ml of normal citrated plasma the clotting time of which is recorded as in the thromboplastin generation test (Note that two concentrations of CaCl are used in this test, M/20 in the incubation mixture and M/40 for the substrate clotting times) At 15 20 and 25 minutes further 0 1 ml samples are removed from the incubation mixture and tested similarly Since 5 minutes elapse between each test all 4 dilutions of plasma can be tested at once Thus the 1/32 dilution is mixed with factor V serum and phospholipid and the $CaCl_2$ is added at 1 minute by the incubation stop watch This mixture will then be tested at 11 16 21 and 26 minutes The 1/64 dilution is mixed with the other reagents and its incubation started at 2 minutes on the incubation stop watch it in turn will be tested at 12 17 22 and 27 minutes Finally the 1/128 dilution is set up at 3 minutes and tested at 13 18 23 and 28 minutes The results of such a test are

shown in Table 49 In this example it will be seen that 20 and 25 minutes appear to be the best incubation times and the 1/16 to 1/128 dilutions the best dilutions to use

The test is carried out on the selected dilutions of normal plasma using two incubation times exactly as described above The abnor-

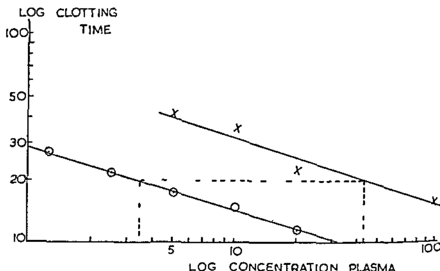


Fig 53 The measurement of antihaemophilic globulin in plasma using the A H G assay method of Biggs Eveling and Richards 1955 The log of the concentration of plasma is plotted against the log of the clotting time in seconds In this example the 1 in 8 dilution of plasma is plotted as 100 per cent concentration.
O——O represents results obtained with dilutions of normal plasma
X——X represent results obtained with the plasma of a mildly affected haemophilic subject
The dotted lines indicate the method of calculating the A H G content when the two lines are reasonably parallel In this example the patient would be said to have 7 5 per cent of A H G

mal (suspected haemophilic) sample is tested similarly using dilutions ½ to 1/16 If it is found that all the dilutions of the abnormal sample give clotting times that are too short (less than 20 sec) then the test must be repeated with higher dilutions (1/32 to 1/128) A control test is included in which the plasma dilution is replaced by 0 1 ml of citrate saline

Normal samples vary greatly in activity and there is much difficulty in selecting a standard We have found that if fresh citrated blood in silicone treated containers is centrifuged at 3000 r p m for ¼ hour to remove platelets the platelet poor supernatant plasma may be stored in silicone treated tubes in 1 ml amounts at −20 C with little deterioration for 2-3 months Using this stored plasma as a standard its value in terms of average normal plasma may be

determined by testing it together with 10-20 freshly collected normal samples

To assess the results the minimum clotting times are plotted against concentration of plasma using double logarithmic paper An approximate straight line is obtained (Fig 53) When figures for an abnormal are similarly plotted a straight line parallel to that of the standard should be obtained and the results may be expressed as percentage of normal (Fig 53)

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